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# **Biomimetic Synthetic Lubricants (Biosurfactants)**

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**Masters of Philosophy**

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**Jan 2014**

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**Summary**

Poly(styrene-co-maleic anhydride) (PSMA) based copolymers are known to undergo conformational transition in response to environmental stimuli. This smart behaviour makes it possible to mimic the behaviour of native apoproteins. The primary aim of this study was to develop a better understanding of the structure-property relationships of various PSMA-based copolymers sought. The work undertaken in this thesis has revealed that the responsive behaviour of PSMA-based copolymers can be tailored by varying the molecular weight, hydrophobic (styrene) and hydrophilic (maleic acid) balance, and more so in the presence of additional hydrophobic, mono-partial ester moieties. Novel hydrophilic and hydrophobic synthetic surfactant protein analogues have successfully been prepared. These novel lipid solubilising agents possess a broad range of HLB (hydrophilic-lipophilic balance) values that have been estimated. NMR spectroscopy was utilised to confirm the structures for PSMA-based copolymers sought and proved useful in furthering understanding of the structure-property relationships of PSMA-based copolymers. The association of PSMA with the polar phospholipid, 2-dilauryl-*sn*-glycero-3-phosphocholine (DLPC) produces polymer-lipid complexes analogous to lipoprotein assemblies present in the blood plasma. NMR analysis reveals that the PSMA-based copolymers are not perfectly alternating. Regio-irregular structures, atactic and random monomer sequence distribution have been identified for all materials studied. Novel lipid solubilising agents (polyanionic surfactants) have successfully been synthesised from a broad range of PSMA-based copolymers with desired estimated HLB values that interact with polar phospholipids (DLPC/DPPC) uniquely. Very low static and dynamic surface tensions have been observed via the du Noüy ring method and Langmuir techniques and correlate well with the estimated HLB values. Synthetic protein-lipid analogues have been successfully synthesised, that mimic the unique surface properties of native biological lubricants without the use of solvents.

The novel PSMA-DLPC complexes have successfully been combined with hyaluronan (hyaluronic acid, HA). Today, the employment of HA is economically feasible, because it is readily available from bacterial fermentation processes in a thermally stable form - HyaCare®. The work undertaken in this thesis highlights the usage of HA in biolubrication applications and how this can be optimised and thus justified by carefully selecting the biological source, concentration, molecular weight, purity and most importantly by combining it with compatible boundary lubricating agents (polar phospholipids). Experimental evidence supports the belief that the combined HA and PSMA-DLPC complexes provide a balance of rheological, biotribological and surface properties that are composition dependent, and show competitive advantage as novel synthetic biological lubricants (biosurfactants).

**Keywords:** Hyaluronan, Poly(styrene-co-maleic anhydride), Langmuir trough, Brewster angle microscopy, NMR spectroscopy.

***To my family***



# Acknowledgements

First of all, I would like to acknowledge my supervisor Professor Brian Tighe for all his help, support, kindness and inspiration. He is an exceptional role model, and an amazing teacher.

I would like to express my gratitude to Dr Val Franklin for all her assistance and support during my MPhil studies. Thanks also go to Dr Mike Perry - the NMR specialist, for all the jokes, and for training me on the NMR facilities at Aston.

I am also grateful for the research funding from EPSRC, Surgicraft Ltd and the Aston Biomaterials Research Unit.

I would like to take this opportunity to convey my sincere appreciation to the entire Aston Biomaterials Research Unit for their patience and for providing me with such an enjoyable time.

Last but not least, words cannot justify, the support from my family and friends, whilst writing this MPhil thesis. Thank you all.

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### ***-List of Abbreviations***

Poly (styrene-co-maleic anhydride)	<b>PSMA</b>
Hydrophilic-lipophilic balance	<b>HLB</b>
Nuclear Magnetic Resonance	<b>NMR</b>
1,2-dilauryl- <i>sn</i> -glycero-3-phosphocholine	<b>DLPC</b>
Hyaluronan	<b>HA</b>
<i>Acute respiratory distress syndrome</i>	<b>ARDS</b>
Neonatal respiratory distress syndrome	<b>NRDS</b>
Osteoarthritis	<b>OA</b>
Surface Active Phospholipid	<b>SAPL</b>
Glycosaminoglycan	<b>GAG</b>
Ophthalmic Viscosurgical Devices	<b>OVDs</b>
Surfactant protein B	<b>SP-B</b>
Surfactant protein C	<b>SP-C</b>
Poly (ethylene glycol)	<b>PE</b>
Tear lipocalin	<b>TL</b>
High density lipoprotein	<b>HDL</b>
1,2-dipalmitoyl- <i>sn</i> -glycero-3-phosphocholine	<b>DPPC</b>
Styrene-maleic acid	<b>SMA</b>
Transmembrane	<b>TM</b>
Conjugate of neocarzinostatin and poly(styrene-co-maleic acid)	<b>SMANCS</b>
Neocarzinostatin	<b>NCS</b>
SMA/lipid particle	<b>SMALP</b>
Gastrointestinal	<b>GI</b>
Phospholipases A2	<b>PLA<sub>2</sub></b>
Ophthalmic Viscosurgical Device	<b>OVD</b>
Cluster Determinant 44	<b>CD44</b>
Extracellular Matrix	<b>ECM</b>
Nuclear Magnetic Resonance	<b>NMR</b>
Magnetic pulse	<b>B<sub>1</sub></b>
Applied field	<b>B<sub>0</sub></b>

### ***-List of Abbreviations***

Polarization Enhancement Nurtured During Attached Nucleus Testing	<b>PENDANT</b>
Hydrogen NMR	<b><sup>1</sup>H-NMR</b>
Carbon NMR	<b><sup>13</sup>C-NMR</b>
Two-dimensional NMR	<b>2D NMR</b>
Hetero Nuclear Single Quantum Coherence	<b>HSQC</b>
Insensitive Nuclei Enhanced by Polarisation Transfer	<b>INEPT</b>
Pascal Second	<b>PaS</b>
Friction force	<b><i>F</i></b>
Normal force	<b><i>N</i></b>
Coefficient of friction	<b><i>μ</i></b>
Osmole	<b>Osm</b>
Platinum-Iridium	<b>Pt-I</b>
Surface pressure	<b><i>π</i></b>
Interfacial tension	<b><i>γ</i></b>
Molecular area	<b><i>A</i></b>
Boltzmann constant	<b><i>K</i></b>
Thermodynamic temperature	<b><i>T</i></b>
Polytetrafluoroethylene	<b>PTFE</b>
Brewster angle microscopy	<b>BAM</b>
Charge coupled device	<b>CCD</b>
Enhanced permeability and retention	<b>EPR</b>
Polysciences	<b>POL</b>
Sigma-Aldrich	<b>S-A</b>
Sartomer	<b>SAR</b>
Chemical shift	<b><i>δ</i></b>
Nitroxide mediated polymerisation	<b>NMP</b>
Reversible addition fragmentation chain transfer	<b>RAFT</b>
Tetramethylsilane	<b>TMS</b>
Isoelectric point	<b>IEP</b>
Critical pH	<b>pH*</b>

### ***-List of Abbreviations***

Hydrophilic-lipophobic balance	<b>HLB</b>
Acid dissociation constant	<b>K<sub>a</sub></b>
Acid dissociation constant at logarithmic scale	<b>pK</b>
Nadia Rasul	<b>NR</b>
High Performance Liquid Chromatography	<b>HPLC</b>
Molecular Weight	<b>MW</b>
Surface tension	<b>ST</b>
liquid/liquid interface	<b>L/L</b>
Liquid/vapour interface	<b>L/V</b>
Solid/vapour interface	<b>S/V</b>
Solid/liquid interface	<b>S/L</b>
Solid	<b>S</b>
Liquid condensed	<b>L<sub>c</sub></b>
Gaseous	<b>G</b>
Collapse pressure	<b>π<sub>c</sub></b>
Surface tension	<b>ST</b>
Standard deviation	<b>SD</b>
Deuterated water	<b>D<sub>2</sub>O</b>
Poly(vinyl alcohol)	<b>PVA</b>
Benzalkonium chloride	<b>BAK</b>
Polyhexamethylene biguanide	<b>PHMB</b>



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# **CHAPTER 1: INTRODUCTION**

## **1.0 Biomimesis and biological lubricants**

Biological systems show remarkable physical properties and have always been a source of inspiration for man-made structures. Biomimesis is a concept of designing materials, resembling biological systems in structure and/or function. The objective of the work in this thesis was to employ a combination of biomimetic strategies and material science, leading towards so-called bio-inspired, synthetic biological lubricants (biomaterials)[1].

The main focus of this thesis was to develop a novel therapy and biosurface treatment for the management of lubricant deficient diseases such as, osteoarthritis (OA) and dry eye syndrome. All biological lubricants have a similar function in that they act as boundary lubricants to prevent focal adhesions and minimise the transmittance of shear forces.

Native biological lubricants, i.e., lung surfactant, tear film and synovial fluid play a crucial role in coating the body's surfaces and transporting materials. These biological films coat the biological surfaces/interfaces. Synthetic mimics to treat such lubricant deficient diseases, should therefore possess similar functions and offer a range of applications to medicine that are not available from current biomaterials.

Before we can mimic the natural biolubrication systems, we need to understand the mechanism by which they operate. The biological fluids also exhibit some remarkable similarities in their composition, in that they all contain a phospholipid (a lipoidal source) in combination with an amphipathic apoprotein, lipocalin (in the ocular environment) to organise the lipid into lamellar sheets. By mimicking the complexes found in native lubricating fluids, it may be possible to replace their physico-chemical properties and offer a completely new treatment modality for lubricant deficient diseases.

The work conducted in this thesis has utilised the Astosomes technology developed by Tighe and Tonge *et al* at Aston University [2].

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Body site	Native lubricant (biological surfactant)	Disease	Existing treatment (Biomaterial)
lungs	lung surfactant	ARDS/NRDS	artificial lung surfactant (Survanta®)
eyes	tear film	dry eye syndrome	artificial tears (lipid spray-Clarymist™)
synovial joints	synovial fluid	osteoarthritis (OA)	synthetic synovial fluid (HA viscosupplementation) (Synvisc/Orthovisc)

Table 1.1. Lubrication deficient diseases and current management (existing treatment).

### 1.1 Lung surfactant

Natural lung surfactant is a mixture of protein and lipids. The bulk of the material (about 90%) is made up of phospholipids, with phosphatidylcholine and in turn dipalmitoylphosphatidylcholine making the majority of the phosphatidylcholines. The other major components are proteins, making up to about 10% of the natural lung surfactant. The lungs essentially consist of a series of minute interconnecting fluid-lined sacks known as the alveoli. This arrangement maximises the surface area for gaseous exchange across a fluid/air interface [2]. Surfactant is concentrated in the smaller alveoli, making the surface tension in the smaller alveoli less than that in larger alveoli. Lower surface tension equalises the pressure amongst different sizes of alveoli and keeps the smaller alveoli from collapsing when the air flows into larger lower-pressure alveoli. With lower surface tension, the work needed to expand the alveoli with each breath is also greatly reduced. This biological surfactant reduces the surface tension at the alveoli surface. Surfactants are molecules that disrupt the cohesive forces between water molecules [1].



Figure1.1.The biological surfactant in lungs  
[[www.respiratorytherapycave.blogspot.co.uk](http://www.respiratorytherapycave.blogspot.co.uk)]

Proper function of pulmonary surfactant depends on the rapid formation of a monolayer, at the air-liquid interface in alveoli and recurring delivery of surfactant to the interface from the subsurface region (subphase). This process is comprised by a number of steps. Bilayer to surface monolayer conversion requires energy and is catalysed by hydrophobic surfactant proteins, SP-B and SP-C. H.W.Taeusch *et al* [3] reports that adding various polymers such as hyaluronan (HA) and polyethylene glycol (PEG) used clinically, enhances elements of surface activity, and improves lung function. This suggests that the polymers aid in the adsorption of biological surfactants. HA is a unique polymer that is secreted as a linear chain not covalently bound to a peptide. It is a non-sulfated, polymeric glycosaminoglycan (GAG). Internal hydrogen bonding of the chain contributes to the complex physical properties of HA. These make the bonds quite rigid, extending the molecule over a large volume and allowing HA binding of up to 1000 times its weight of water. A variety of mechanisms may explain how HA and PEG increase surfactant adsorption. Investigators have suggested that hydrophobic surfactant proteins enhance surfactant adsorption by catalysing the disruption of the surfactant vesicle bilayer and the formation of a monolayer at the air-subphase interface. Perhaps HA would affect adsorption. Protection from inactivation conveyed by polymers may also occur because the depletion is strong enough to overcome (to a degree) the electrostatic and steric repulsion imposed by a surface active inhibitor from the adsorbed on the interface. The increased surfactant adsorption helps to drive the inhibitor from the interface, preventing inactivation. Ionic interactions between charged surfactant



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proteins, surfactant lipids, and charged polymers may also affect rates of adsorption, a mechanism relevant to HA but not to PEG [3]. Theoretically, the anionic or hydrophobic character of HA may allow it to interact with proteins including SP-B that carries a net positive charge at physiological pH [3].

### **1.2 Tear film**

The native tear film is a dynamic structure. It consists of a superficial lipid layer, an intermediate aqueous phase and a mucus layer. The lipid layer is important in retarding the evaporation of the aqueous layer and stabilising the tear film. Phospholipid micellar structures are believed to provide the boundary lubrication. Tear lipocalin (TL), an apoprotein complexed with other tear components, may also contribute to the high, non-Newtonian viscosity of the tear film and its low surface tension, features that are essential for tear film stability [4-7].



Figure 1.2. The tear film; the proposed multilayer structure [4].

In the lipid deficient dry eye state, the boundary lubrication is impaired and artificial tears are administered to provide relief from ocular discomfort/dryness. TL is a member of the lipocalin protein family. It is thought to act as a lipid scavenger and an aid to tear film stability. It is known to bind a broad array of lipids including cholesterol, fatty acids, phospholipids and glycolipids, and therefore has a potential role in tear viscosity. It is one of the most abundant tear proteins. The lipid binding properties of TL are implicated in all of its functions. TL scavenges lipids from the corneal surface and delivers them into the aqueous phase of tears. It may also have an important role in removing lipids from the corneal surface to maintain the wettability and integrity of the ocular surface [4-7].

### **1.3 Synovial fluid**

Synovial fluid is found in all articular joints and is responsible for lubrication during low impact joint movement and shock absorbing properties during high impact activities. The synovial membrane secretes synovial fluid, which forms a thin film over the surfaces within the articular capsule. It is a slippery, weight bearing film that reduces the coefficient of friction between articular cartilages. The mechanism by which it lubricates is by a weeping lubrication. It consists of HA, surface active phospholipids (lipoidal material), proteins, mucins, proteoglycans and interstitial fluid filtered by the blood plasma. It contains a phagocytic cell that removes microbes and the debris that results from normal wear and tear of the joint [2, 8-10].



Figure 1.3. A typical synovial joint.

HA is secreted by the fibroblasts like cells in the synovial membrane and the interstitial fluid filtered from blood plasma. Synovial fluid also contains phagocytic cells that remove microbes and the debris that results from normal wear and tear in the joint. When a synovial joint is immobile for a time, the fluid is quite viscous (gel-like), but as joint movement increases, the fluid becomes less viscous. Synovial joints include the ball-and-socket joints (e.g., hip) and hinge joints (e.g., interphalangeal). They possess a cavity and permit the opposed cartilaginous articular surfaces to move painlessly over each other. Movement is restricted to a required range, and stability is maintained during use. The load is distributed across the surface, thus preventing damage by overloading or disuse. The fibroblasts

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release HA into the joint space, which helps to retain fluid in the joint. Synovial fluid is a highly viscous fluid secreted by the synovial cells and has a similar consistency to plasma. Glycoproteins ensure a low coefficient of friction between the cartilaginous surfaces. In joint disease conditions such as arthritis the synovial fluid is dysfunctional, no longer lubricates the synovial joint effectively [2, 8-10].

### **1.4 Lubrication mechanisms**

Biological lubricants reduce the coefficient of friction. This is a scalar value, which describes the ratio of the force of friction between two bodies and the force pressing them together. Low coefficients of friction usually suggest comfort.

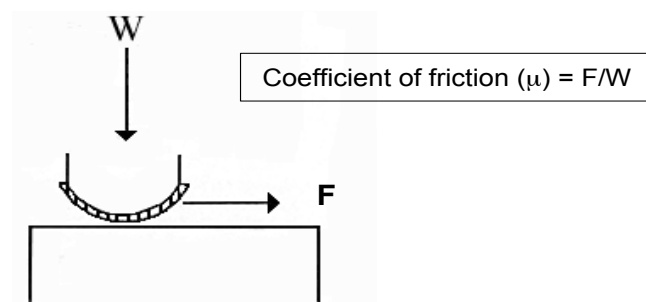


Figure 1.4. The coefficient of friction

There are three regimes that can be identified that describe lubrication, characterised by the degree of contact between the two solid surfaces, I) fluid film/hydrodynamic no contact is made; the lubricant separates the two surfaces apart, II) boundary lubrication mechanism (fluid film is displaced) and the III) the mixed lubrication regime where partial contact is made (see Figure 1.5) for a picture of all three regimes.

In the boundary lubrication regime the lubricant film thickness approaches zero, as the lubricant is adsorbed onto the surfaces, thus, there is a high degree of contact between the two solids. In the hydrodynamic lubrication/fluid film regime, the film thickness is greater than the combined surface roughness of both surfaces; hence the surfaces are completely separated from each other. For this type of lubrication the friction of coefficient rises with an increase of the viscosity of the lubricant and or speed but decreases as the load increases. In between is the mixed lubrication regime where both the boundary and fluid film mechanisms act simultaneously, either supporting or competing with each other. The average film thickness is less than the combined surface roughness of both surfaces, thus, there is partial contact between

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the asperities of the two surfaces. In this case the applied load is supported by the asperity contact and the hydrodynamic pressure forces.



Figure 1.5. Lubrication mechanisms characterised by a degree of contact (Stribeck curve) [[www.ewp.rpi.edu/hartford/~ernesto/F2012/FWM/Pics/Lubrication/Stribeck](http://www.ewp.rpi.edu/hartford/~ernesto/F2012/FWM/Pics/Lubrication/Stribeck)].

To achieve a low coefficient of friction (a mixed lubrication regime) both hydrodynamic and boundary lubrication mechanisms need to be in operation simultaneously in competition and supporting each other (see Figure 1.5 above). Existing commercial treatment to manage OA is a viscosupplementation injection of HA. Contemporary literature suggests that although HA is a major component of synovial fluid, it is not solely responsible for the lubricating capability of synovial fluid. HA provides hydrodynamic lubrication, as it is viscoelastic. Hydrodynamic lubrication is when the lubricant separates the two surfaces apart and no contact is made. The lubrication mechanism by which synovial fluid operates, is in the mixed lubrication regime. In this lubrication mechanism, the hydrodynamic and boundary lubrication mechanisms occur simultaneously. The lubrication mechanisms are in competition and support each other, in order to achieve the remarkable low coefficient of friction exhibited in healthy synovial joints. However, much evidence supported by B.A Hills *et al* [11-21] infers that it is the SAPL, that imparts the thin hydrophobic outermost lining to the normal articular surface, and is the boundary lubricant reducing friction to remarkable low levels. D.W.Nitzan, *et al* [22] also concluded that it was reasonable to say that HA plays an important indirect role in the steady state of the lubrication process of joints by protecting SAPL from being lysed

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by phospholipase PLA<sub>2</sub>. Recent literature suggests that lubricin and HA and surfactant proteins help to solubilise and orientate the lipoidal material, at the articular surface [22].

### **1.5 Biological lubricants and their common features**

The common features in the composition of natural lung surfactant, tear film and synovial fluid are, SAPLs, apoproteins (surfactant proteins), lipoidal matter, mucins, proteoglycans and glycoproteins (lubricin). Upon reflection of the literature review conducted, it implies that nature uses a similar mechanisms and that the likely presence of complexes formed from interactions between proteins and lipids within the lung, eye and at articular joints suggests that by prefabricating lamellar structures, coherent films can be readily adsorbed in the short time periods encountered, as these interfaces rapidly form and reform, e.g., during breathing in the lung, blinking in the eyes and articular joint movement at synovial joints. Such films render the biosurfaces lubricious and wettable while at the same time imparting a low surface tension. The dynamic surface activity of most biological fluids, including tears, lung surfactant and synovial fluid is a result of spatially specific interactions between lipids and proteins in which the lipid is orientated by a protein into a pre-assembled lamellar form that can be readily adsorbed at the biological interface. Lack of such assemblies results in the lubricity deficiency diseases of which dry eye is one example. In an attempt to mimic these endogenous assemblies and produce a synthetic material to treat such diseases we have identified responsive polymers. These are based on styrene-maleic acid copolymers, which adopt a surface active conformation, at precise levels of ionization, and behave in a similar manner as apoproteins, the ability to solubilise film forming phospholipids and produce structures that closely resemble native lipid-apoprotein assemblies.

### **1.6 Astosomes and their biomedical applications**

Astosomes are lipid-containing compositions developed at Aston by Tighe and Tonge *et al* [2, 23]. They can be primarily employed in the field of biochemistry and medicine. Astosomes are useful surfactants and solubilising agents for certain substances. They are especially useful as they produce aqueous solutions of substances that are lipid soluble. These lipid-containing compositions provide artificial surfactants having useful applications in medicine, e.g., as lubricating formulations for medical use. There is a need for lubricating surfactants to lubricate

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surfaces of medical devices and prostheses, e.g., artificial joints and contact lenses that are fitted in human or animal body. These solutions are lipid-containing compositions, which consist of substantially clear solutions containing a membrane-forming polar lipid and a synthetic amphipathic polymer. The polymer must contain both hydrophobic groups and anionic hydrophilic groups acting as a lipid-solubilising agent, which interacts with, and solubilises the lipid in the aqueous medium. The preferred embodiments will usually comprise a phospholipid and the synthetic amphipathic polymer with which it is combined will have a balance of hydrophobic and anionic hydrophilic groups evenly arranged along a linear backbone. The solubilisation effect described in the literature referred to was attributed to break-up and reorganisation of the vesicles structures accompanying conformational changes occurring in the polymer upon lowering of the pH, leading to the formation of lipid/polymer complexes producing small micellar discoidal particles or assemblies [1, 23,26]. The lipids employed are polar - they have a highly polar head portion attached to a nonpolar hydrophobic tail, and are generally composed of a pair of relatively long hydrocarbon chains, such that in aqueous media the lipid molecules tend to associate and form membrane structures at interfaces, possibly as lipid monolayers or bilayers. It is believed that in aqueous media, at least over a particular pH range, the solubilising synthetic amphipathic polymers specified will generally adopt a helical coil configuration with the hydrophobic side groups presented along the a surface facet and the anionic hydrophilic groups presented along the opposite facet. It is believed that they interact with the lipid in the aqueous medium to form discoidal micellar particles or assemblies of sub-liposomal dimensions in which the lipid forms a bilayer core[23,26]. Responsive hydrophobically associating polymers change their shape according to the degree of charge and collapse from an extended chain to adopt a 'secondary structure' in which their hydrophobic and hydrophilic groups at opposite facets of an  $\alpha$  helical coil form an amphipathic structure. When hypercoiling polymers are combined with film-forming lipids they associate to produce lipid-polymer complexes analogous to lipoprotein assemblies such as high density lipoprotein HDL. These polymer-lipid complexes are highly surface active and possess an ability to solubilise poorly water soluble drugs into the lipoidal core to render them aqueous soluble. The hypercoiling polymers offer the required functionality. As a result of their ability to interact with phospholipids and form nanostructures of analogous size to those of native high density lipoproteins, the hypercoiling polymers offer unique advantages in developments of pharmaceutical nanotechnology necessary for delivery of drug and genetic material to particular cellular and intracellular destinations. In summary,

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hypercoiling polymers can be used to mimic the behaviour of some of the functions that account for the essential living processes, and may therefore, be suited for application to living systems and in particular to biomedicine. They have considerable potential for use in drug delivery systems and as synthetic apoproteins. The polymer-lipid recombinants synthesised at Aston (Astosomes) can mimic the lipocalin-lipid complexes found in the native system and hence perform a similar function. The outermost lipoidal film is the protective boundary lubricant [2,23, 26].



Figure 1.6. Cryo TEM electron micrographs of poly(maleic acid styrene) DLPC vesicles - 3120,000 showing axial (left) and lateral (right) views of nanostructures found by Tighe and Tonge *et al* at Aston University [23].

### **1.7 The design of novel biosurfactants (biological lubricants)**

The basic concept behind the use of polymer/lipid-based complexes is that these materials are highly lubricious and mimic the body's own method of lubricating the joints with sheets of phospholipid or fatty material. It is thought that they will act as 'molecular ball bearings' to minimise friction at the surface of a damaged arthritic joint and thereby are likely to reduce pain experienced by patients. In addition, these materials associate at surfaces and so are likely to be retained for a longer period than conventional treatment modalities, and as a consequence are likely to give the patient a longer period of relief from pain. This represents one of the first practical applications of the emerging science of nanotechnology to the area of human medicine. Employing the Astosomes technology involves a novel approach for solubilising lipids. As Astosome formulations are water soluble so can be easily combined with HA-based solutions. This may be a direction forward in formulating a biomimetic synthetic lubricant, such as a synthetic synovial fluid and an artificial tear. The ultimate aim of the work conducted in this thesis was to examine the potential of using novel poly(styrene-co-maleic acid) based - polar phospholipid complexes to improve the efficacy of HA-based lubricants. This was undertaken by employing the Astosomes technology, developed by Tighe and Tonge *et al* at Aston University [2, 23,26].

# **CHAPTER 2:**

# **LITERATURE REVIEW**



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### **2.0 Introduction**

The focus of the work at Aston has been to produce polymer-based complexes similar in structure to the protein-based lipid assemblies found at fluid interfaces within the body. These play a crucial role in coating the body's surfaces and transporting materials. Such synthetic mimics should therefore possess similar functions to those of native lipoproteins and offer a range of applications to medicine that are not available from current biomaterials. The ultimate aim of the work presented in the thesis is to design and develop novel biosurfactants, based on hyaluronan (HA), synthetic surfactant protein analogues and polar phospholipids, as a unique treatment, to manage lubricant deficient diseases [2, 23-26].

#### **2.0.1 Overview**

This literature review chapter introduces the various studies undertaken in this thesis. Background information regarding the topics of the upcoming results chapters is captured here. It begins with discussing the biomedical applications of styrene-maleic anhydride copolymers, in section 2.1. The main focus of this section is to identify that PSMA-based copolymers can be utilised, as synthetic protein analogues. Furthermore, these materials can be refined to synthesis polyanionic surfactants, as polar phospholipid solubilising agents. Section 2.2, describes the role of polar phospholipids in biolubrication, and highlights the challenge to deliver them to lipid deficient sites. Furthermore, the role of HA, surfactant proteins and lubricin in relation to this is also discussed. The role of HA as a biolubricant, is discussed in section 2.3, with particular reference to its role in ophthalmic and orthopaedic biolubrication applications.

Finally, section 2.4 concludes the future directions for this topic of research based on the literature collected and suggests areas of investigation towards the development of unique biosurfactants, for biolubrication applications.

#### **2.1 Biomedical applications of PSMA based copolymers**

Polymers such as proteins, polysaccharides, and nucleic acids are present as basic components in living organic systems. Synthetic polymers that are designed to mimic these biopolymers have been developed into very active fields; due to their industrial and scientific value. They have been progressively developed from the description of the unique properties of biopolymers. It is of utmost importance to

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understand how protein structure is related to aspects to biological function, so we can design synthetic protein analogues that mimic native protein function[27-36].

### **2.1.1 Responsive copolymers**

Response to stimulus is a basic process of living systems. Based on the lessons from nature, scientists have been designing useful materials that respond to external stimuli such as temperature, pH, light, external field, chemicals and ionic strength. The responses are manifested as dramatic changes in one of the following: shape, surface characteristics, solubility (which this work is concerned with), and formation of an intricate molecular self assembly. These stimuli could be classified as either physical or chemical stimuli. Chemical stimuli, such as pH, ionic factors and chemical agents, will change the interactions between polymer chains or solvents at the molecular level. Two or more signals could be simultaneously applied in order to induce response, in so-called dual responsive polymers systems. This dynamic aspect of natural macromolecules is an essential characteristic of all living systems. If biomaterials science is to mimic biological systems more effectively, we must harness macromolecules that possess such dynamic behaviour. pH-responsive polymers consist of ionisable pendant groups that can accept and donate protons in response to the environmental pH. There are two types of pH-responsive polyelectrolytes; weak polyacids and weak polybases. The representative acidic pendant group of weak polyacids is the carboxylic groups. The balance between hydrophobic association and electrostatic repulsion is crucial [23-26].

### **2.1.2 PSMA copolymers as synthetic protein analogues**

PSMA-based copolymers are known to undergo conformational transition in response to environmental stimuli. PSMA is a class of pH-responsive, hydrophobically associating polymer that changes its shape according to the degree of charge and collapses from an extended chain to adopt a 'secondary structure' in which hydrophilic and hydrophobic groups occupy separate domains and so form a surface active polymer. This behaviour mimics that of native apoproteins that arrange their hydrophobic and hydrophilic groups at opposite facets of an alpha helical coil, to form an amphipathic molecule [23].

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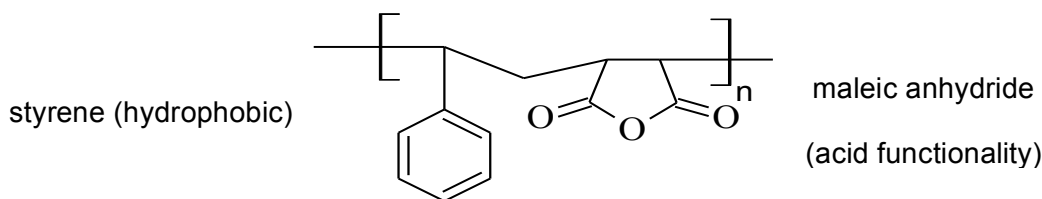


Figure 2.1. Repeat unit of PSMA (full anhydride).

The ability of certain polymers to hypercoil or to associate hydrophobically to form excessively compact molecules offers one possible mechanism by which macromolecules could be made to change their conformation, and therefore, their function, in response to local stimuli. A polymer with weakly charged pendant groups, i.e., either weak acids or bases, forms an extended structure as a result of mutual repulsion between charged groups. If, in addition, the polymer also bears alkyl pendant groups, then the latter will be subject to hydrophobic interactions which will tend to restrict the alkyl side chains to a minimal volume within an aqueous environment, thereby, allowing maximal hydrogen bonding to occur between the water molecules. This so-called, 'hydrophobic effect', is the principal driving force in the formation of lipid-based assemblies such as those found in biological membranes, and in defining the conformation of native proteins. This conformational change occurs according to variation in environmental pH [43-44]. The driving forces for this conformational change are due to a balance of electrostatic repulsion between charged subgroups, (charged maleic acid groups), Van der Waals cohesion of uncharged side groups, hydrogen bonds and interactions with the solvent and ions. The two most important effects in controlling polymer conformation are charge and the hydrophobic effect [23-24].



(A) **Hydrophobic - styrene/partial esters**

Figure 2.2. (A) Apoprotein-apolipoprotein III lateral view showing five amphipathic alpha helices - surfactant behaviour. (B) Cartoon of amphipathic coil showing hydrophobic (red) and hydrophilic (blue) facets of a synthetic apoprotein [23].

Styrene pendant groups also provide a hydrophobic moiety and combine with maleic anhydride (hydrolysed to maleic acid) to form a pseudo alternating hypercoiling copolymer. The term hypercoiling was used by Dannhauser to describe the behaviour of styrene/maleic acid copolymer. Previously studies by Ferry had observed that PSMA-based copolymers exhibited an intrinsic viscosity some four times less than that predicted by the Flory equation, as assessed by viscosity measurements conducted in dilute aqueous acid under theta solvent conditions. In a polymer solution, a theta solvent (or  $\theta$  solvent) is a solvent in which polymer coils act like ideal chains, assuming exactly their random walk coil dimensions therefore in a good solvent, the Mark-Houwink equation exponent is  $1/2$ . Thermodynamically, the excess chemical potential of mixing between a polymer and a theta solvent is zero

In a non-ionic theta solvent, viscometric data indicated that the polymer adopted a random coil conformation, which under acidic conditions was found to contract its linear dimensions by a factor of two. Investigations by potentiometric titration and dilatometry also indicate a pH-induced conformational transition, characteristic of a hypercoiling polymer [23].

There is a need to solubilise and characterise stable and active membrane proteins. Timothy J. Knowles *et al* [36] report how monodispersed lipid disks formed by styrene-maleic acid (SMA) copolymers preserve the integrity of transmembrane (TM) proteins and form biocompatible, thermostable, and soluble nanoparticles, for their biophysical analysis in a lipid environment [36].

Amphipathic polymers including SMA adsorb on and destabilise membranes via pH-dependent conversion from extended chains to secondary structures [23]. In the presence of lipids they form polymer/lipid assemblies such as nanometer-sized disks. A proprietary polymer/lipid assembly termed "Lipodisq" has been developed by

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Malvern Cosmeceutics Ltd as a delivery vehicle for hydrophobic pharmaceutical agents. They integrate an  $\alpha$  - helical bundle and  $\beta$  -barrel TM protein within a SMA/lipid particle (SMALP). This advance provides a simple and effective method of solubilising TM proteins from liposomal fractions and potentially from bilayers without requiring detergents. SMALP offer advantages over other solubilising agents such as lipoproteins, amphipols or PreserveX detergents (Tebu-Bio), as the lipid environment is maintained and detergents are not needed, consequently native structure and bound lipids can be retained [36].

SMANCS; a conjugate of neocarzinostatin and poly(styrene-co-maleic acid), is a polymer-protein conjugate that was the first polymer-protein conjugate to be brought to market. The conjugate contains two polymer chains of styrene-co-maleic anhydride (SMA); a 15000 (g/mol) material is used, covalently bound to the anti-tumour protein neocarzinostatin(NCS). This increases the lipid-solubility and enables the administration of SMANCS in the phase-contrast agent Lipiodal, which increases plasma half-life, allows tumour visualisation and improves the degree of tumour targeting. PSMA is an ideal compound to modify NCS because of the anhydride group, which would react with the amino group and because the styrene side chain would confer potential hydrophobicity to the molecule. The PSMA type employed has 30-50% of the maleic acid in the reactive anhydride form and half of the free carboxyl groups are butylated; this design was found to give substantial hydrophobicity. The nature of the alkyl group and the extent of alkyl esterification of the carboxyl groups are important factors in determining the binding affinity of SMANCS to albumin, its lipid solubility and the biological function of the covalently attached drug. The degree of polymerisation of styrene-maleic acid and the different types of (maleyl carboxylate ester) alkyl groups (such as *n*-butyl, ethyl, or methyl) and the free carboxyl ester itself, significantly affect the binding affinity of the conjugate to albumin. SMANCS with free carboxyl SMA do not bind albumin. SMANCS is capable of activating macrophages, natural killer cells and T-cells and it induces interferon- $\gamma$ . When the abilities of different types of SMA (e.g.butyl, ethyl and free maleyl residues) to activate macrophages were compared, the *n*-butyl ester was chosen as the most desirable alkyl residue [37-42].

PSMA will not be rapidly hydrolysed in the blood plasma and hence will be more likely to deliver any drug to the target site before degradation of the micelle and loss of its contents. In addition, PSMA lacks allergic and pharmacological potential of non-native peptides or proteins [1, 26].

### **2.1.3 Synthesis of polyanionic surfactants (lipid solubilising agents)**

PSMA-based copolymers are insoluble in water. The first challenge is to render them water soluble. This is achieved by manipulating the maleic anhydride functionality. PSMA is an amphiphilic molecule and possesses an anhydride ring, which opens upon hydrolysis to form two carboxylic groups. In the presence of excess base, hydrolysed styrene-maleic anhydride resins perform as classic anionic polymeric surfactants, combining hydrophobic (styrene) and hydrophilic (carboxylic) structural units along a common backbone.

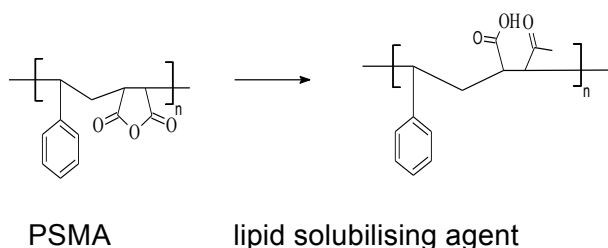


Figure 2.3. Synthesis of lipid solubilising agents.

Changing the styrene-maleic anhydride ratio in the base resin will have an obvious impact on the balance of hydrophobic/hydrophilic properties and thus the pH-responsive behaviour. This is achieved by two strategies; by increasing the hydrophobic component (styrene units) and from esterification of the maleic anhydride moieties. This will essentially modulate pH-responsive behaviour.

### **2.1.4 Chemistry of hydrolysed PSMA solutions**

The structure of the hydrolysed PSMA-based molecule is pH dependent. The different structures of PSMA as a function of pH and are presented in Figure 2.4. This observation, presented in Figure 2.4, confirms the model proposed by Malardier-Jugroot *et al* [43], using a more direct method.



Figure 2.4. The different structures of styrene-co-maleic anhydride copolymers in various environmental pH [43].

At pH 3 and 10, the 90° angle between two monomers induces the interlaced-orthogonal structure of the backbone of SMA. The shuttle-like association was successfully observed by TEM with the size about 200-400 nm in the latexes. At pH 7, the chains of SMA seem to be linear; rod-like micelles were obtained with lengths of about several microns. Another explanation could be the effect of the mixed surfactants templates. It is well known that the geometry of the surfactant plays a crucial role in the aggregation of the surfactants in solution. Israelachvili *et al* and Kumar and Mittal *et al* defined a packing parameter ( $P$ ) to estimate the aggregate structures formed from surfactant in aqueous solution: which has crucial role on the aggregation of the surfactants in solution.



Figure 2.5. TEM images of the micelles of SMA at different pH values: A. pH = 3; B. pH=7; and C. pH=10 [43].

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The study of water conformation around hydrophilic and hydrophobic parts of styrene-maleic anhydride has been carried out by C.M.Jugroot *et al* [43]. The molecules possess a hydrophobic and hydrophilic group which allows the determination of the influence of the water orientation around the hydrophilic and hydrophobic groups. The hydrophilic interactions were found to be direct and long range. Whereas, the hydrophobic interactions were found to be indirect and short range. The models proposed by C.M.Jugroot *et al* [43] have been defined to represent the close interactions and hydrogen bonds between the hydrophilic part of SMA and the water molecule.[43-44].

### **2.2 The role of surface active phospholipids (SAPLs) in biolubrication**

For many years it was assumed that HA was the joint lubricant, as it is a major component of synovial fluid and had been credited with being responsible for the low coefficient of friction exhibited by articular cartilage. However, much evidence supports that (in particular work carried out by B.A.Hills *et al* [11-21] the surface active phospholipid (SAPL), which imparts the thin hydrophobic outermost lining to the normal articular surface, is the boundary lubricant reducing friction to remarkable low levels. Even though HA solutions are slippery and HA is the major component of synovial fluid, it is not the boundary lubricant. Basically it possesses no load bearing capabilities. Oligolamellar layers of SAPL adsorbed to the articular cartilage surface have been observed by electron microscopy in the outermost hydrophobic layer, which imparts superb lubrication, reducing wear and lowering friction to physiological levels that are extremely low by engineering criteria. SAPL is also an effective releasing agent capable of inhibiting articular gelling/fusion implicated in stiffness. Although, SAPL can be administrated ostensibly as a lubricant, it can have other physiological benefits, including putative roles in control of cartilage hydration and scavenging free radicals implicated in chondrocyte destruction [11].

It is a fundamental requirement of any substance acting as a boundary lubricant that it must first be adsorbed or otherwise bound to the surface, before it can impart lubrication. The stronger the binding and the more cohesive the adsorbed lining, the better is the lubrication and resistance to wear under load [11-21]. The lipid content of cartilage amounts to 0.3-4%. Lipid is composed of three basic components, cholesterol, triglycerides, and phospholipids. However, in the normal joint, and in the lung, the major component is phospholipids. The major sub-fraction of phospholipid is phosphatidylcholine, which is very surface active. Thus with strong adsorption of SAPL molecules and strong cohesion of this adsorbed lining, SAPL satisfies the two



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fundamental criteria for a high-load-bearing boundary lubricant. It explains how a hydrophilic phase such as protoglycan can be rendered so hydrophobic [11-23]. SAPLs offer much potential for providing boundary lubrication (the outer most part of a biofilm) *in-vivo* for several reasons. They are found *in-vivo* adjacent to the moving surfaces of the body in forms in which they are available in a surface active form and, moreover, have been shown to display the same multilayer adsorption to the pleural surface as to other epithelial surfaces [11]. Most possess polar moieties, which readily bind to the negative sites on most mucosal and other epithelial surfaces to affect strong adsorption. Cations interspersed in the plane of the phosphate ions will neutralise those ions, effectively rendering the phospholipids cationic, i.e., and the 'pseudocationics' with characteristically strong adsorption. The fatty acids in the hydrophobic moieties are mostly of ideal chain length for lubrication. When adsorbed via their polar moieties these long hydrocarbon chains are orientated and packed in much the same way as described above for free fatty acids and the lubrication is as good but the monolayer is more tightly bound and, therefore, more durable on the surfaces. They contain a phosphate group towards the middle of the molecule, which is ionised at physiological pH for most locations *in-vivo*. These ions provide an ideal point for pulling neighbouring molecules together if small cations are placed between them, thus imparting strong cohesion, in addition to adhesion [11-21]. Many other researchers have also reported that SAPL is indeed the boundary lubricant in nature and reported evidence to support this conclusion. [45-48]. Polar phospholipids such as 1,2-dilauroyl-*sn*-glycero-3 phosphocholine (DLPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) are present in synovial fluid, lung surfactant, and the tear film, but at different concentrations [11-21]. They are saturated and thus are able to pack tightly and allow organised self-assembly at the biological interface. See Figure 2.6 for the chemical structure of these polar phospholipids.

It has been indicated that adsorbed phospholipid can rupture the fluid film if one stops blinking. One explanation is that an adsorbed layer of phospholipid could act as second line of defence in providing lubrication at the points of high load-bearing where the fluid layer is inadequate - as occurs with mucopolysaccharide solutions. Boundary lubrication could be a major factor in the comfort of contact lenses where the 'high spots' could penetrate any fluid layer. Other places where the same system might apply are the gastrointestinal (GI) tract where phospholipids extracted from gastric juice have been shown to be good boundary lubricants. It is highly likely that boundary lubrication imparted by surfactants of some form is providing a back up to

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fluid film lubrication imparted at points of high load and it is quite likely that those surfactants are phospholipids. In the articular joint the SAPLs are the load-bearing lubricant. For example, when we stand up, it is the SAPLs that act as the boundary lubricant, protecting the articular cartilage. In the ocular environment the lipid layer retards the evaporation of the aqueous layer. In the lungs the SAPLs, aid breathing [11-21]

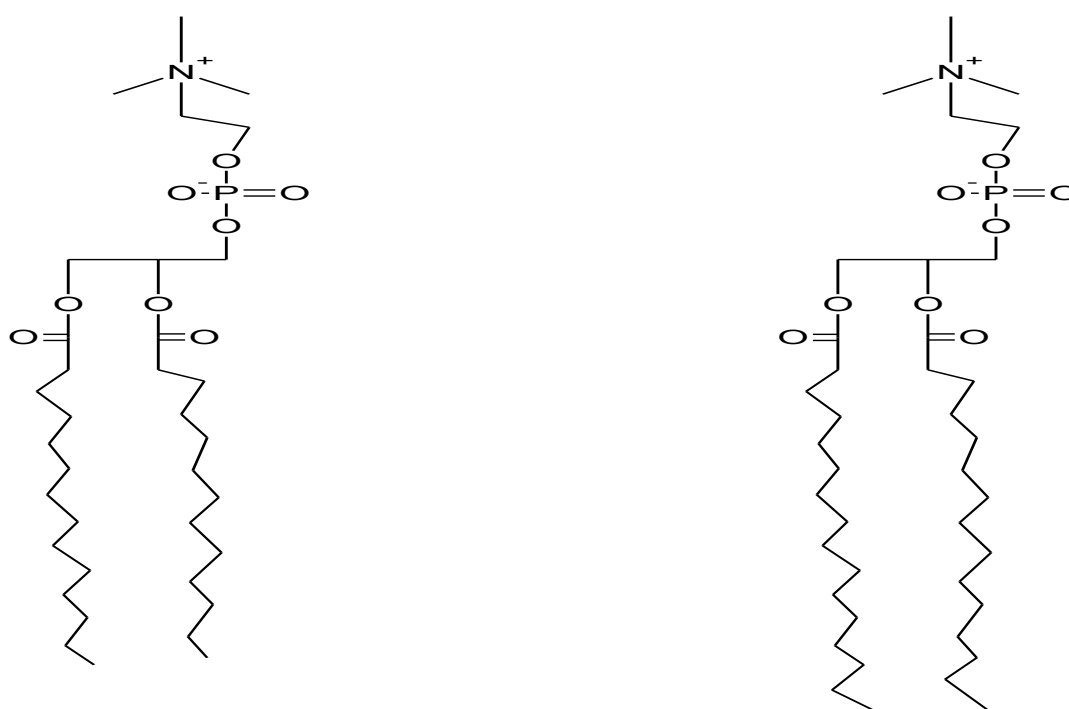


Figure 2.6. Chemical structure of 1, 2-dilauroyl-*sn*-glycero-3 phosphocholine (DLPC) and 1, 2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) utilised in this study.

So, overall, we can conclude that the SAPLs play a crucial role in the biological lubrication mechanism. They are simply nature's boundary lubricating agents. The term 'surfactant' is used in the physical sciences to denote any amphipathic substance (complex) which lowers the surface energy. The whole complex of which consists of protein, carbohydrate and lipids; collectively referred to as the biological surfactant [11-21, 45-48].

### 2.2.1 The interactive role of SAPLs with HA in biolubrication

D.W.Nitzan *et al* [22] concluded that it was reasonable to say that HA plays an important indirect role in the steady state of the lubrication process of joints by protecting SAPL from being degraded by phospholipase PLA<sub>2</sub>. However, as excessive loading generates free radicals within joint (among other effects), the HA that is degraded in this way is incapable of protecting SAPL from lysis by PLA<sub>2</sub>. When the rate of degradation exceeds that of synthesis, there will be insufficient

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replacement of HA and/or SAPL, resulting in denudation of the articular surfaces. These are then exposed to increasing friction, and hence increased danger of degenerative joint changes. The model suggests that PLA<sub>2</sub> is secreted into the extracellular fluid, where it constitutes an active component in the inflammatory process, posing a threat to the cell membrane phospholipids of the target cells. It has been shown that a mechanism similar to that described for SAPL in this study acts at the cellular level; cell-surface proteoglycans protect the cell membrane from being lysed by PLA<sub>2</sub>, a condition that is abrogated in the presence of reactive oxidative species. The latter degrade cell-surface proteoglycans, rendering the membrane phospholipids accessible to hydrolysis by PLA<sub>2</sub> [22]. In conclusion, although HA has no load-bearing capability, it has an indirect role of protecting the SAPL. HA and SAPL are the two most abundant active ingredients in synovial fluid. It is believed that the major properties of synovial depend on them and their interactions. Therefore, HA plays an important role in joint lubrication, even though *in-vitro* studies suggest, that it is not the load bearing boundary joint lubricant [22, 49-53].

### **2.2.2 The role of surfactant proteins in biolubrication**

Four proteins have been found, known universally as SP-A (surfactant protein A), SP-B, SP-C, and SP-D. Of these, SP-A and SP-D are hydrophilic, while the other two are very hydrophobic. Surfactant proteins are very sensitive to experimental conditions (temperature, pH, concentration, substances such as calcium, and so on). SP-A was the first surfactant protein to be identified, and is also the most abundant. Its molecular mass varies from 26-38 kDa. It can be found in an open or closed form depending on the other substances present in the system. Calcium ions produce the closed-bouquet form. SP-A is necessary for the production of tubular myelin, a lipid transport structure unique to the lungs [2]. Tubular myelin consists of square tubes of lipid lined with protein. At 43 kDa, SP-D is the largest surfactant protein. SP-D is a dodecamer. SP-B and SP-C are directly involved in protein re-spreading. However, they bind preferentially to anionic lipids. SP-B is 8.7 kDa and it consists of alpha helices with disordered links. One model for its structure suggests that two sets of positive helices border two neutral domains. SP-C is the smallest surfactant protein, at 4.2 kDa. It is also one of the more abundant; estimates for the ratio of SP-B to SP-C range from 1:2 to 1:10. SP-Cs main structural feature is an alpha-helix, exactly the right length to span a lipid bilayer. It has been suggested that SP-C can act as a lever to move lipids. The hydrophobic SP-B and SP-C are tightly bound to phospholipids. These proteins play important roles in maintaining the surface

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tension-lowering properties of pulmonary surfactants [3, 53-60]. The hydrophobic proteins, SP-B and SP-C, play a key role in modulating the arrangement of surfactant phospholipids into bilayer and monolayer structures, and are instrumental in the transfer of surface active materials between the different structural assemblies. [2,54-60].

### **2.2.3 The role of lubricin in biolubrication**

Is lubricin the lubricant or is lubricin the macromolecular water-soluble carrier for the otherwise highly insoluble SAPL. The argument is that analysis has shown how SAPL makes up 12% of lubricin, which would render this macromolecule an ideal carrier for SAPL, while phospholipids also bind to HA, which has similar protein chains [11]. The classic experiment of Radin *et al* [21] led them to seek a protein unique to synovial fluid, and they finally isolated one in form of a proteoglycan with a molecular weight around 335, 000. Because this compound imparted the remarkable lubricating capabilities of synovial fluid, they termed it lubricin. They identified 86% of these macromolecules, but of the remaining 14%, 12% was identified as the SAPL, raising the question whether lubricin is the lubricant or simply the macromolecule; it must therefore render that surface very hydrophilic, that is, as hydrophilic, as the underlying proteoglycan. The normal articular cartilage is very hydrophobic. SAPL entities are small molecules, which bind to amino acid groups that comprise the protein chains in proteoglycans such as lubricin. The other feature of joint phospholipid is that it is largely saturated and therefore more surface active, such as 1, 2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), because the fatty acid chains of the nonpolar moieties can pack together more tightly when they are straight. Thus, with strong adsorption of SAPL molecules and strong cohesion of this adsorbed lining, SAPL satisfies the two fundamental criteria for a high-load-bearing boundary lubricant. The problem with formulating exogenous SAPL as an injectable substance to replenish deficiency in the joint is its extremely low solubility in water. This can be overcome by using a water-miscible solvent such as propylene glycol. Alternatively, a macromolecular water-soluble carrier could be used, such as HA or gelatine which contains amino acid groups to which phospholipids can bind reversibly. Lubricin and maybe other proteinaceous macromolecules in synovial fluid, play an important role in the joint as the carrier for the otherwise highly insoluble SAPL, but are not the lubricant per se. Another factor emphasised elsewhere is that the outermost layer of a surface imparts boundary lubrication, and so how could binding of such a hydrophilic, water-soluble substance as lubricin ever render the articular surface so hydrophobic. The load-bearing boundary lubricant is

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surely SAPL the same lubricant found on other sliding surfaces *in-vivo*. It has been theorised that lubricin interacts with hydrophobic surfaces, articular cartilage and latex. Lubrication may be provided by apposed and pressurised hydrophilic moieties, and it is these mucinous glycoproteins that have amphipathic behaviour. Synovial SAPL, if present naturally, may play some role in joint lubrication by rendering articular cartilage hydrophobic, through some as yet undiscovered means. This would enable hydrophobic-hydrophobic attraction (in an aqueous environment) of the lubricant onto its surface. Resolution of whether lubricin is the lubricant or its carrier will depend on analyses for elemental phosphorus in purified lubricin [21]. If elemental analysis revealed substantial phosphorus this would imply that the role of Lubricin was the macromolecular water-soluble carrier for the otherwise highly insoluble SAPL [61-64].

### **2.3 Glycosaminoglycans (GAGs)**

The extracellular spaces, particularly those of connective tissues such as cartilage, tendon, skin, and blood vessel walls, consist of collagen and elastic fibers embedded in a gel-like matrix known as ground substance. Ground substance is composed largely of glycosaminoglycans (GAG), unbranched polysaccharides of alternating uronic acid and hexosamine residues. Solutions of GAGs have slimy, mucus like consistency that results from their high viscosity and elasticity [2,8-10].



Figure 2.7. Chemical structure of GAG molecules [1].

### **2.3.1 Hyaluronan (HA)**

Hyaluronic acid (also known as hyaluronan) is an important component of synovial fluid (the fluid that lubricates the joints), and the vitreous humour of the eye. HA molecules are composed of 250-25,000  $\beta$  (1-4) - linked disaccharide units. The anionic character of its glucuronic acid residues causes HA to bind tightly to cations such as  $K^+$ ,  $Na^+$ , and  $Ca^{+2}$ . HAs structural features suit its biological function. Its high molecular weight and numerous mutually repelling anionic groups make HA a rigid and highly hydrated molecule which, in solution, occupies a volume  $\sim 1000$  times that in its dry state [8-10].

### **2.3.2 Hyaluronan as a biolubricant**

HA is a high molecular weight, unbranched, glycosaminoglycan (GAG) found in all mammals. It is a unique and highly versatile biopolymer. GAGs are made from repeating disaccharide repeating units containing a derivative of an amino sugar, either glucosamine or galactosamine. At least one of the sugars in the repeating unit has a negatively charged carboxylate or sulfate group. Chondroitin sulfate, keratin sulfate, heparin, heparin sulfate, dermatan sulfate, and HA are the major GAGs. GAGs are usually attached to proteins to form proteoglycans [65-72]. Proteoglycans more closely resemble polysaccharides than proteins, in that the carbohydrate makes up as much as 95% by weight of the biomolecule. Proteoglycans function as lubricants and structural components in connective tissue, mediate adhesion of cells to the extracellular matrix (ECM), and bind factors that stimulate cell proliferation. HA plays a vital role in embryonic development, ECM homeostasis, wound healing, and tissue regeneration. Therefore, understanding the biology and chemistry of HA is of utmost importance [65-72]. Biomaterials made from derivatised and crosslinked HA have received a great deal of attention in the bioengineering community particularly with respect to orthopaedics, ophthalmology, dermatology, and in general applications such as tissue engineering and drug delivery. HA is naturally derived, nonimmunogenic and also has multiple sites for modification and inherent biological activities; therefore, this unique biopolymer has great potential for creative applications in biomedical engineering [65-72].

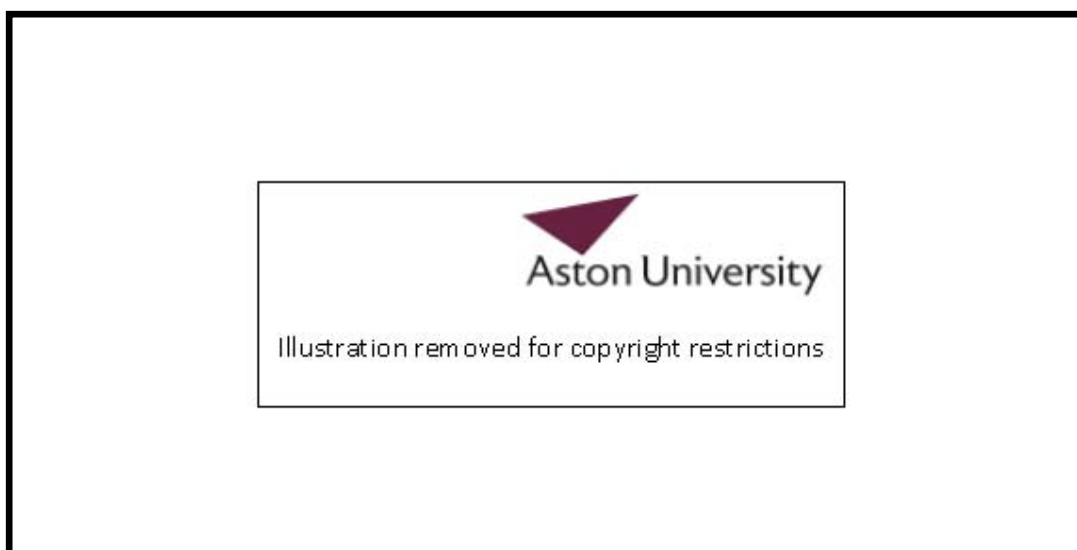


Figure 2.8. Chemical structure of HA; an alternating copolymer of N-acetyl glucosamine and *D*-glucuronic acid - a non-sulphated GAG.

See Figure 2.8 above, for the chemical structure of HA, identifying its main functional groups (carboxyl, hydroxyl and acetamido). HA is a naturally derived polymer composed of disaccharide repeats of *D*-glucuronic acid and *N*-acetyl glucosamine. Each disaccharide repeat unit of HA contains three possible modification sites: the hydroxyl, carboxyl, and acetamido groups, respectively. This relatively simple structure is conserved throughout all mammals, suggesting that HA is a biomolecule of considerable importance. In the body, HA occurs in salt form, hyaluronate, and is found in high concentration in several soft connective tissues including skin, umbilical cord, synovial fluid and vitreous humour. Notable amounts of HA are also found in lung, kidney, brain, and muscle tissue. In commercial production, HA has been extracted from rooster comb and human umbilical cord or manufactured in large quantities and, more recently, by bacterial fermentation. The cellular synthesis of HA is a unique and highly controlled process. Most GAGs are made in the golgi apparatus HA, however, is synthesised at the plasma membrane and immediately extruded out of the cell and into the extracellular matrix (ECM).[65]. This process is carried out by a group of proteins called HA synthases, which are located in the cell membrane. HA is unique amongst the GAGs, as it does not contain any sulphate and differs from other proteoglycans, in that it is not found covalently attached to proteins. [65-71].

The structure of HA imparts unique physicochemical and biological properties that depend on molecular weight. When extracted from tissues, HA is polydisperse with an average molecular weight of several millions. In physiological solution, HA exists as a stiffened random coil. HA of  $1 \times 10^6$  Da (2650 disaccharide repeats) has a contour length of 2.5 $\mu$ m. Secondary hydrogen bonds form along the axis of HA,

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imparting stiffness and creating hydrophobic regions that allow the formation of ordered structures. HA solutions are highly viscoelastic; in other words, when shear rate is increased, the HA chains align in the direction of flow, resulting in a decreased solution viscosity. This shear thinning can be seen when delivering HA through a syringe. HA is a highly hydrophilic polymer. Each *D*-glucuronic acid unit contains one carboxyl group, giving rise to HAs polyanionic character, at physiological pH. Because of this property, HA is thought to play several roles in the ECM including space filler, lubricant and osmotic buffer. By forming a hydrated polymer network, HA can act as a sieve, restricting the movement of pathogens, plasma proteins, and proteases. In addition, HAs polyionic structure is able to scavenge free radicals, which mediates inflammation by imparting an antioxidant effect. HA occupies a large hydrated volume and therefore shows solute-solute interactions, at low concentrations. HA solutions show excluded volume effects, as it restricts access to other macromolecules. HA is a polyelectrolyte and therefore the HA solution properties were also greatly affected by ionic strength. Experimental data supports the view that intramolecular hydrogen bonds and polyanionic properties of HA both contribute and provide a highly expanded macromolecular conformation. The most plausible explanation for the large hydrodynamic volume of HA and hence other non-Newtonian viscoelastic properties is the presence of multiple dynamic hydrogen bonds between adjacent saccharides. This restricts rotation and flexion at the glycosidic bonds and creates a stiffened yet mobile polymer chain. The flexibility and permeability properties of the HA network can then be accounted for in terms of inter-chain hydrodynamic interactions of this extended structure, with entanglements being especially important at elevated concentrations. In a narrow range of acid pH around pH 2.5, HA does have different properties and these may reflect chain-chain association. In contrast, HA properties in the broad pH range 3-8 are incompatible with any significant intermolecular self-association that forms stable tertiary structures. At physiological pH the properties of HA solutions can be predicted directly from the behaviour in dilute solutions and even at very high concentrations, individual chains remain mobile and they undergo no transition to a gel-like state. In dilute solution HA behaves as a stiffened random coil [65-68].

HA has been widely utilised in viscosupplementation treatment to manage the pain associated with osteoarthritis. Table 2.1, lists some commercial products available in the market that have been employed as viscosupplementation injections *in vivo*.



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Table 2.1. Some commercial viscosupplementation HA products.

<b>Generic name</b>	<b>Concentration (MW)</b>	<b>Supplier</b>	<b>Biological source</b>	<b>Articular joint</b>
Synvisc (Hylan G-F20)	(Hylan A = 60,000 & Hylan B) 15mg/ml	Genzyme	chicken comb	knee
Orthovisc	15mg/ml	Anika Therapeutics, Surgicraft	rooster comb	knee
Fermatron	1% (w/v) (1 million D <sub>a</sub> )	Smith & Nephew	<i>streptococcequi</i>	synovial joint
Supartz	25mg/2.5ml	Smith & Nephew	avain	knee
NeoVisc	1% solution (0.8 million D <sub>a</sub> )	Steller pharmaceuticals	rooster comb	knee, shoulder, Hip
Hyalgan	10mg/ml 0.5-0.73 kD <sub>a</sub>	Sanofia Aventis	rooster comb	
Artzal	0.5-1.0 kD <sub>a</sub>	Seikagaku corporation	rooster comb	knee
Suplasyn	500-730 kD <sub>a</sub>	77 Canada pharmacy	non-avain	synovial joint
Viscoseal	0.5 % solution	TRB Chemedica	bacterial fermentation	post arthroscopic surgery
Durolane	20 mg/ml	Smith and Nephew	non-animal derived	knee
Arthrease	1% solution	Savient pharmaceutical	non-avain	knee
Adant	25mg/2.5ml	Tramedico	avian	knee

### **2.3.3 HA viscosupplementation**

The worldwide osteoarthritis (OA) market is forecast to grow steadily, and to reach 7 billion dollars by 2015. Viscosupplementation is an approach to manage articular pain associated with OA. Commercial treatment is an injection of crosslinked HA. HA plays a vital role in the development of cartilage, the maintenance of the synovial, and the regeneration of tendons. High concentrations of HA can be found in the ECM of all adult joint tissues, including the synovial fluid on the outer cartilage. In part because of its viscoelastic nature and ability to form hydrated matrices. HA acts in the joint as a lubricant and a shock absorber. In diseases such as OA, the concentration and molecular weight of the HA naturally present in the joint are decreased, contributing to stiffness and pain. Viscosupplementation treatments aim to treat these conditions, and to this end, a variety of HA materials have been successfully applied as clinical therapies. Researchers have also investigated cross-linked HA as cell delivery scaffolds for cartilage and bone tissue engineering. Esterified HA (Hyaff, Fida Advanced Biopolymers) supports the growth of chondrocytes and osteoblasts. OA is the most common form of chronic arthritis worldwide. HA and hylan products provide an opportunity to treat OA. Viscosupplementation is an intra-articular (IA) therapeutic modality for the treatment based on the physiological importance of HA in synovial joint. To date, all HA-based viscosupplement injections are highly concentrated HA in buffer and some are crosslinked. Preliminary human clinical studies were performed in the early seventies using the non-inflammatory fractions of HA (NIF-Na HA). Synvisc sold by Biomatrix (New Jersey, USA) is available in Canada and Sweden and has been launched in the UK. Orthovisc, sold by Anika Therapeutics (USA) is marketed in Canada, the Netherlands, and Turkey. Hyalgan, available from Fida, Italy, has been launched in Canada, South East Asia, Italy, France, Germany, and some South America countries [65-68].

The mode of action of viscosupplementation to dysfunctional synovial fluid appears to be debatable due to the rapid clearance of HA from synovial joints after IA injection together with the long-term symptomatic benefits experienced by patients. Subsequent laboratory investigation showed that HA exhibited analgesic/anti-inflammatory, cytoprotective and immunoregulatory activities, many of which were mediated through specific cell receptors such as CD44. The pharmacological activities of HA provided an explanation of how this agent could produce symptomatic relief in OA. *In-vitro* studies using chondrocytes or cartilage explant cultures have suggested a chondroprotective role of HA. Cartilage protection may

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occur, not from a direct affect on the chondrocytes, but secondary due to regulation of cytokine, free radical, and protection activities in the synovial fluid and synovium, which act on chondrocytes and promote matrix catabolism. However, the long lasting clinical benefits can also be reconciled by acceptance that this agent is capable of pharmacologically modifying certain pathologic pathways in OA that give rise to symptoms. In this regard, the partial restoration of synovial cell metabolism and normalisation of HA biosynthesis ("viscoinduction"), which has been shown in both in animal models and human subjects using has within the MW range of  $0.5 \times 10^6$  -  $1 \times 10^6$  Da, may be of considerable importance.

### **2.3.4 Ophthalmic applications of HA**

HA ophthalmic products are growing rapidly. HA has been used in Ophthalmic Viscosurgical Devices (OVDs) since 1979 [70]. HA was first isolated from the vitreous humour by Karl Mayer in 1934 [70]. It was then incorporated into artificial tears. More recently the introduction of HA into contact lens materials - Safe-Gel™, (Filcon IV-1-day Safe-Gel, Filcon 1B - 7-Day Safe-Gel™ and methafilcon A-Biocal™ 1-Day), contact lens packing solutions, and a now a multipurpose solution (Safe-Gel Hyal™) means that the importance to optimise its performance in ophthalmic applications has heightened. HA, a natural major component of the vitreous humour of the eye, has found many successful applications in ophthalmologic surgery, because, in part, it forms viscoelastic and highly hydrated matrices. It is particularly useful as a space filling matrix in the eye: thus intraocular injection of HA during surgery is used to maintain the shape of the anterior chamber. Furthermore, HA solutions also serve as a viscosity enhancing component of eye drops and an adjuvant to eye tissue repair. High molecular weight (MW) polymers with water binding properties and high viscosity have increasingly been used as hydrogels for ophthalmic applications. Firstly, in artificial tears, they soothe, protect and lubricate the ocular surface, thus relieving the symptoms of dry eye syndrome. Secondly, in solutions for contact lens wearers, they also provide better and prolonged comfort to contact lens wearers through moisturising, lubrication and reduction of the blink frequency. To overcome some of the limitations of previously employed polymer materials in ophthalmic solutions, growing attention has thus been paid to non-Newtonian and particularly to pseudo plastic polymers such as HA. With topical ophthalmology, this biocompatible, non-immunogenic and biodegradable polymer is well recognised and considered as the 'next generation comfort ingredient'. The unique competitive advantage of HA solutions for ophthalmic applications is that HA solutions are non-Newtonian and shear thinning. The high viscosity at low shear

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rates (when the eye is open) and low viscosity at high shear rates (during blinking) thus allowing an even distribution of the solution and lubrication of the ocular surface.

Another important feature of high molecular weight and anionic biopolymer is its muco-adhesivity, which provides effective coating and long-lasting protection of the cornea as well as extended residence times on the ocular surface. Finally, when topically instilled on the eye, HA has shown to promote physiological wound healing by stimulating corneal epithelial migration and proliferation of keratocytes and to reduce the healing time of corneal epithelium [69-72]. In 1979, the first HA based OVD was patented (Healon<sup>®</sup>) and many more followed. Below are some of the reasons why HA is being increasingly used in ophthalmic biomaterials.

- Cellular healing - CD44
- Biocompatible / Non immunogenic
- Mucoadhesive
- Viscoelastic, non-Newtonian, shear thinning, pseudoplastic
- Wetting agent
- HyaCare<sup>®</sup> - available in a ultra pure, cheaper and thermally stable form
- HA is a component of vitreous humour.

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Table 2.2. Classification of some OVDs [70].

<i>HA based OVD</i>	<i>Content</i>	<i>MW (D)</i>	<i>V<sub>0</sub> (mPAS)</i>
<b><i>Higher viscosity cohesive / viscoadaptive</i></b>			
MicroVisc Phaco <sup>TM</sup>	2.5% NaHa	7.9M	24.0M
Healon GV <sup>TM</sup>	2.3% NaHa	4.0M	7.0M
<b><i>Superviscous cohesive</i></b>			
Microvisc Plus <sup>TM</sup>	1.4% NaHa	7.9M	4.8M
Healon GV <sup>TM</sup>	1.4% NaHa	5.0M	2.0M
<b><i>Viscous - cohesive</i></b>			
MicroVisc <sup>TM</sup>	1.4% NaHa	6.1M	1.0M
Provisc <sup>TM</sup>	1.0% NaHa	2.0M	280K
Healon <sup>TM</sup>	1.0% NaHa	4.0M	230K
Biolan <sup>TM</sup>	1.0% NaHa	3.0M	215K
Amvisc <sup>TM</sup>	1.2% NaHa	1.0M	100K
Amvisc Plus <sup>TM</sup>	1.6% NaHa		100K
<b><i>Medium viscosity dispersive</i></b>			
Viscoat <sup>TM</sup>	3.0% NaHa/4%CS	500K	41K
Vitrax <sup>TM</sup>	3.0% NaHa	500K	25K

### **2.3.5 Ophthalmic Viscosurgical Devices (OVDs)**

OVDs are commonly used in anterior segment to facilitate surgery and enhance safety. They create space, stabilise tissue, pressurise, and protect delicate endothelial cells. Because of the numerous and varied OVDs available for ophthalmic surgery, they must be classified to logically select what is optimal to manage a particular problem. They are commonly classified on their rheological properties, which relates most closely to ophthalmic use: zero-shear viscosity and cohesion. The viscosity of the OVDs is a function of its molecular weight, concentration, and the shear force to which it is exposed. High molecular weight mass, long molecular chains tend to entwine, making the entire solution move as a whole mass [69-72].

### **2.4 Concluding remarks and future directions**

Current literature review and clinical trials suggest that perhaps HA [8-10,65-68], lubricin [45-48], SAPLs [11-21] and surfactant proteins [49-53] are interactively responsible for the biological lubrication mechanisms. What can be concluded from this is that nature uses similar mechanisms. Overall, lipids are insoluble in aqueous media, strong evidence in the literature indicates that the interaction of lipids with proteins in nature renders them aqueous soluble through hydrophobic interaction [6]. Therefore, current scientific understanding strongly supports the view that biomimetic biolubricants used to treat lubricant deficient diseases, should be based on synthetic protein-lipid complexes.

Upon review of the literature reported, a few studies will be conducted in order to contribute towards the development of unique biosurfactants (biomimetic synthetic lubricants). This is the main objective of the experimental work conducted in this thesis.

Chapter 4 will discuss the current understanding of PSMA-based copolymers and highlights the need to tailor the design of novel lipid solubilising agents based on the fact that it is vital to attain PSMA-based structures with specific surface properties. NMR spectroscopy will be conducted to better understand the structure-property relationships of these copolymers identified. This will be useful in furthering our ability to refine the structures of PSMA-based copolymers towards the development of effective lipid solubilising agents, at the target physiological conditions.

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Chapter 5 will describe the surface properties of the novel lipid solubilising agents developed. The Langmuir trough technique (with use of a Wilhelmy plate balance), and the du Noüy ring static surface tension measurement will be used to explore the association behaviour of PSMA, both at the interface and in the bulk of a solution. Furthermore, some frictional and rheological behaviour, in relation to this will also be discussed. Novel hydrophilic and hydrophobic synthetic surfactant proteins will also be synthesised from hydrolysing various PSMA-based copolymers sought.

Two major studies will thus focus on designing, synthesising and characterising novel lipid solubilising agents, based from PSMA-based copolymers as synthetic surfactant protein analogues.

Chapter 6 will describe in full details how the novel technology (as introduced in section 1.6 - Chapter 1, page 32) developed at Aston (Astosomes), which allows the solubilisation of polar phospholipid (boundary lubricating agents) by employing novel lipid solubilising agents (without the usage of solvents). This approach overcomes the problems associated with other attempts of lipoidal supplementation, which involve solvents that carry toxicity and components that contain allergic risks.

Langmuir and Brewster angle microscopy techniques will be used for the first time to study these novel biosurfactants developed. Existing commercial attempts for Lipoidal supplementation will also be investigated and the surface properties of these products assessed. The surface properties of a native ocular Lipoidal film (extracted from a worn balafilcon A lens) studied, using Langmuir/BAM will also be discussed for the first time. Furthermore, the advantages of incorporating HA into the biosurfactants developed will also be addressed. This is strongly supported with contemporary reported literature from HA researchers. The novel biosurfactants will be developed (with and without HA). The *in-vitro* surface, rheological and frictional properties of these biosurfactants, will be investigated and reported.

Finally, Chapter 7 with reference to *in-vitro* data obtained, suggests a rational approach for HA employment, as opposed to the compulsive choice of using HA in orthopaedic and ophthalmic lubricity applications.

**CHAPTER 3:  
MATERIALS, EXPERIMENTAL  
METHODS AND TECHNIQUES**



## **-Chapter 3-**

### **3.0 Introduction**

This chapter provides detailed information and pictures regarding the materials, experimental methods and techniques utilised in this thesis. Table 3.1 lists all the materials used in chapters 4-7. The main instrumental techniques employed in the experimental work are;

- Nuclear Magnetic Resonance (NMR) spectroscopy
- Rheometry
- Frictional studies
- Osmometry
- du Noüy ring method for measuring static surface tension
- Langmuir and Brewster angle microscopy

Section 3.2 describes the NMR experiment and NMR techniques employed during the PSMA NMR (chapter 4) and the HA  $^1\text{H}$ -NMR analysis work (chapter 7). Section 3.3 provides background theory concerning rheology and provides details about the technique used to investigate the rheological behaviour of the respected solution (biolubricant) under study. Section 3.4 highlights the science of biotribology and then defines how the Aston CSM Nano Scratch Bio-tribometer setup is operated, in order to investigate the performance and effectiveness of the solution (biolubricant), under interest. Sections 3.5, 3.6 and 3.7 briefly outline the osmometry undertaken in chapter 7, the countless pH measurements noted throughout the thesis, and the freeze drying methodology adopted for preserving the biosurfactants developed, respectively. Sections 3.8 - 3.10 deals with all the surface chemistry theory and the surface measurement experimental details undertaken in this MPhil thesis.

### **3.1 Materials**

All materials were used as purchased without further purification and are summarised Table 3.1.

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Table 3.1. List of materials utilised in this thesis.

<b>Material</b>	<b>Mw/Purity</b>	<b>Suppliers</b>
PSMA	1,600-350,000	
HA	99%	Sigma-Aldrich
1M NaOH		Sigma-Aldrich
0.1 M HCl		Sigma-Aldrich
DPPC/DLPC	99%	Genzyme pharmaceuticals
Orthovisc	Autoclaved	Surgicraft Ltd/
blink <sup>TM</sup> Contacts eye drops		(AMO)
Focuz Aquify Comfort eye drops	FDA approved	Ciba Vision
Vizualize eye drops	FDA approved	Peach pharmaceuticals
Avizor eye drops		
etafilcon A	FD approved	Vistakon
nelfilcon A		Ciba vision
galafilcon A		Vistakon
lotrafilcon B		Ciba Vision
Ciba Saline		Ciba Vision
HypoTears <sup>®</sup>		Ciba Vision
unpreserved HypoTears <sup>®</sup>		Ciba Vision
1-day Safe-Gel <sup>TM</sup>		Safilens
7-Day Safe-Gel <sup>TM</sup>		Safilens
Hyal Safe-Gel <sup>TM</sup>		Safilens
balafilcon A		B&L
Methanol		Fisher
Chloroform		Sigma-Aldrich
Clarymist <sup>TM</sup>		Eye logic

## 3.2 Nuclear magnetic resonance spectroscopy (NMR)

The object of the NMR experiment is to measure the frequency of a nuclear resonance. The NMR spectrometer is comprised of a strong, highly stable magnet in which the sample is placed and surrounded by transmitter/coils. Additional coils known as shims, are also placed around the sample to counteract variations of field

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gradients. By using these shims the field is made as homogeneous as possible. Remaining inhomogeneities are minimised by spinning the sample to counteract field gradients and render the field as perfectly homogeneous as possible. Spinning the sample tube about its axis means that the sample molecules experience average fields. Very well defined frequencies, and excellent resolution of close, narrow resonances, are obtained in this way. A magnetic pulse  $B_1$  is produced by a gated (switched input) power amplifier driven by a stable, crystal-controlled continuous oscillator. The nuclear signals following the radio frequency,  $B_1$  pulse are then amplified and detected in a device which compares them with the crystal oscillator output ( $B_1$  carrier) and gives a low-frequency, time-dependent output containing frequency, phase and amplitude information. The output is digitised and collected in a computer memory for frequency analysis using a Fourier transform program, and the spectrum that results a function of frequency can be displayed and the resonance frequencies listed [73-79].

#### **3.2.1 One-dimensional NMR spectroscopy**

The approach to any structural problem should invariably start with the acquisition of one-dimensional spectra. Since this provides foundation for further work, it is important that these are executed correctly and full use is made of the data they provide before more extensive and potentially time-consuming experiments are undertaken. A Bruker Avance 300 MHz spectrometer using a 5mm probe head was utilised for all the NMR experiments. All chemical shifts were measured relative to tetramethylsilane, which was set at 0ppm. All spectra were processed using the Avance 3.5 XWIN NMR software. The NMR spectra were plotted using 3.5 XWIN plot, as required.



Figure 3.1. Crossed coil geometry in an NMR probe  $B_0$  acts along the z-axis, the excitation ( $B_1$ ) is delivered along the x-axis, and the receiver coil along the y-axis [73].

### **3.2.2 $^1\text{H}$ -NMR Spectroscopy**

$^1\text{H}$ -NMR spectra were acquired using the simple direct-detect NMR experiment. The sequence consisted of the relaxation delay ( $t_{\text{rod}}$ ), and an efficient pulse (typically  $\pi/4$  or  $45^\circ$ ).

#### $^1\text{H}$ -NMR pulse sequence

$^1\text{H}$     $t_{\text{rod}}$  -  $\pi/4$    Acquire

### **3.2.3 Edited $^{13}\text{C}$ NMR (PENDANT) spectroscopy**

The Edited  $^{13}\text{C}$ -NMR PENDANT pulse sequence (Polarization Enhancement Nurtured During Attached Nucleus Testing) was employed to assign the types of carbons. This technique circumvents the problem of 'lost quaternary carbons' while giving maximum signal intensity by polarisation transfer [78-79].

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#### PENDANT pulse sequence

$^1\text{H}$	$\pi/2 (+x)$	$\pi (+x)$	$\pi/2 (-y)$	$\pi (+x)$	decouple				
$^{13}\text{C}$	$\pi/2(+x)$	$1/4J$	$\pi (+x)$	$1/4J$	$\pi/2(-y)$	$5/8J$	$\pi (+x)$	$5/8J$	acquire

#### 3.2.4 Two-dimensional NMR (2D NMR)

The term dimension in NMR actually refers to the number of frequency dimensions of domains present in a spectrum. For example, a one-dimensional spectrum has one frequency domain but actually contains a second dimension depicting intensity of the signal. Two-dimensional NMR spectra are presented on two orthogonal frequency axes, each analogous to one-dimensional spectrum with the final third orthogonal dimension depicting intensity. They may be presented in different formats, often as contours plots rather than illustrating the intensity dimension, which sometimes complicates their view [80-83].

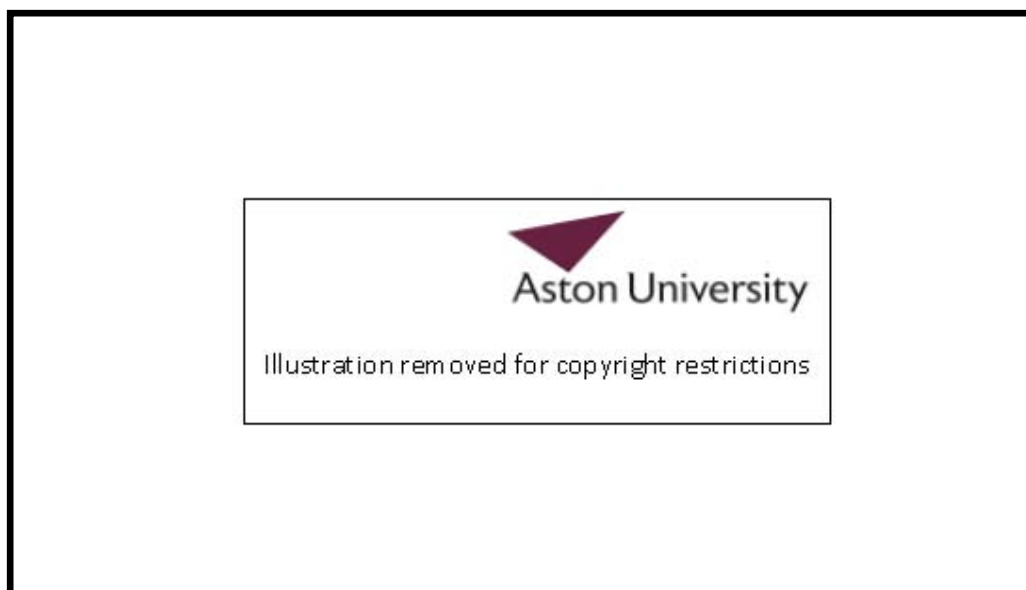


Figure 3.2. Schematic representation of a two-dimensional spectrum [80].

##### 3.2.4.1 2D HSQC spectroscopy

Hetero Nuclear Single Quantum Coherence (HSQC) spectroscopy provides the short-range  $^1\text{J}_{\text{CH}}$  connectivities and thereby applies only to those C atoms which are linked to H and not to non-protonated C atoms. The experiment uses the Insensitive Nuclei Enhanced by Polarisation Transfer (INEPT) sequence to generate transverse x magnetisation which evolves and is then transferred back to the proton by an INEPT step in reverse.

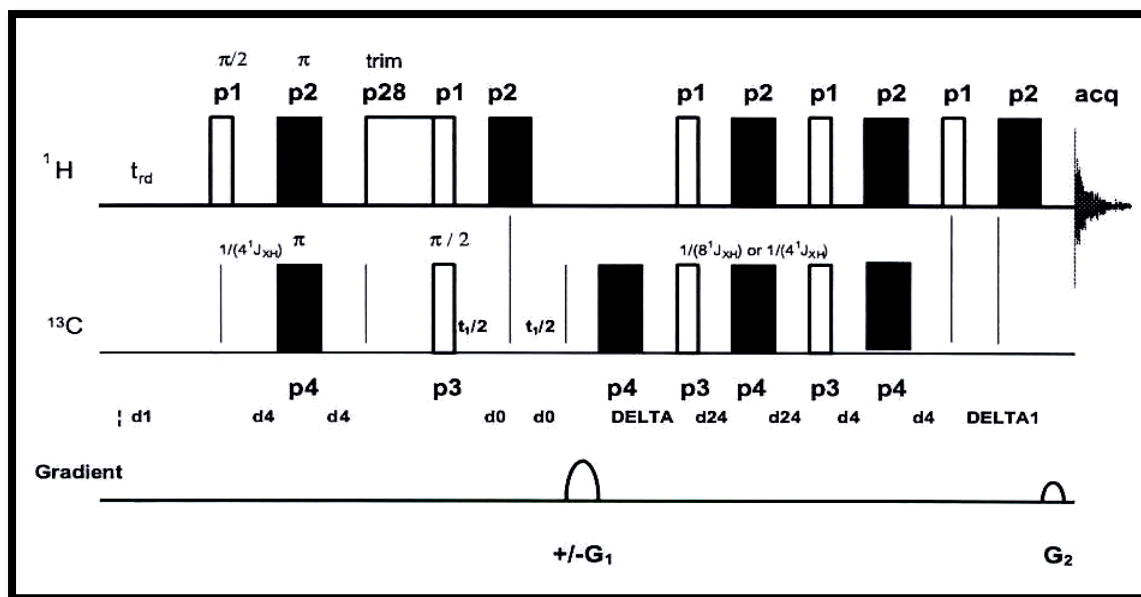


Figure 3.3. HSQC pulse sequence.

### 3.3 Rheology analysis

Rheology is the study of deformation and flow of matter. The viscosity of a material is its resistance to flow and elasticity is the property that enables materials to return to their original dimensions after an applied stress has been removed. Most materials have a combination of both viscous and elastic components hence they are viscoelastic. Viscoelastic properties provide information on how the material is likely to flow under an imposed constant (or cyclic) shear stress or rate. During rheological analysis, the viscoelastic properties of the material are assessed by applying a torque and deforming the fluid with an applied stress causing a strain. Shear stress is defined as the force applied divided by the area of the fluid, shear strain is the displacement of the fluid caused by the force divided by the gap height and shear rate is the rate of change of strain as a function of time. As flow resistance is proportional to force and displacement, viscosity is the quotient of shear stress and shear rate, which is ;

$$\text{Viscosity} = \text{Shear stress} / \text{Shear rate} \text{ Nm}^{-1}\text{S (PaS)} \quad \text{Equ 1}$$

The units of viscosity are  $\text{Nm}^{-1}\text{S}$  and are known as Pascal Seconds (PaS).

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If a material has a viscosity, which is independent of shear stress, then it is referred to as an ideal or Newtonian fluid. The mechanical analogue of a Newtonian fluid is a viscous dashpot, which moves at a constant rate when a load is applied [86].

$$\text{Shear stress} = \text{Force/Area (Pascal Second, PaS)} \quad \text{Equ 2}$$

A few drops of the liquid under investigation are placed on the lower plate of a Bohlin CVO50 rheometer and the viscosity is measured as a function of shear stress, using a CP1°/20mm upper plate [86].

The measured viscosity of a fluid can be seen to behave in one of four ways when sheared, namely:

1. Viscosity remains constant no matter what the shear rate (Newtonian behaviour).
2. Viscosity decreases as shear rate is increased (shear thinning behaviour).
3. Viscosity increases as shear rate is increased (shear thickening behaviour).
4. Viscosity appears to be infinite until a certain shear stress is achieved (Bingham plastic).



Figure 3.4. Bohlin CVO50 rheometer utilised in this thesis [86].

Over a sufficiently wide range of shear stress it is often found that the material has a more complex characteristic made up of several of the above flow patterns. Since it is the relationship of shear stress to shear rate that is strictly related to flow we can directly show the flow characteristics of a material by plotting shear stress vs shear rate. A graph of this type is called a flow curve. The behaviour of materials can often be described by some form of rheological model. Most materials will start to

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deviate from these relationships over a sufficiently large shear range. They are well suited to studying materials over a small shear range or where only a simple relationship is required. The Newtonian flow curve is the simplest type of flow where the materials viscosity is constant and independent of the shear rate. Newtonian liquids are so called because they flow according to the law of viscosity as defined by Sir Isaac Newton [86].

$$\sigma = \gamma * \eta^*$$
Equ 3

Shear stress = shear rate \* Viscosity

Many non-Newtonian materials undergo a simple increase or decrease in viscosity as the shear rate is increased. If the viscosity decreases as the shear rate is increased the material is said to be shear thinning or pseudo plastic. The opposite effect is known as shear thickening. Often this thickening is associated with an increase in sample volume; this is called 'dilatency'. The power law is a good for describing a materials flow under a small range of shear rates. Most materials will deviate from this simple relationship over a sufficiently wide shear rate range [86].

$$\sigma = \eta^* \gamma^n$$
Equ 4

Shear stress = viscosity \* shear rate<sup>n</sup>

Where 'n' is often referred to the power law index of the material. If n is less than 1, the material is shear thinning, if n is more than 1 the material is shear thickening. Polymer solutions, melts and some solvent-based coatings show Power law behaviour over limited shear rates. The viscosity of a material usually decreases with an increase in temperature, assuming no physical/chemical changes are being induced by the applied heat energy. The viscosity of Newtonian liquid decreases with an increase in temperature approximately in line with the Arrhenius relationship. This model describes a materials variation in viscosity with absolute temperature.

#### **3.4 Frictional analysis**

Friction is a force that impedes sliding. The magnitude or 'level' of friction can be expressed in terms of the coefficient of friction. The relationship between friction force ( $F$ ), the normal force ( $N$ ) and the coefficient of friction ( $\mu$ ) is ;

$$\mu = \text{friction force } (F) / \text{normal force}(N)$$
Equ 5



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### **3.4.1 The coefficient of friction**

The coefficient of friction is a scalar value, which describes the ratio of the force of friction between two bodies and the force pressing them together. The coefficient of friction depends on the materials used. It is also important to discriminate between sliding (dynamic) friction and static friction. For sliding friction, the force of friction does not vary with the area of contact between the two objects. This means that sliding friction does not depend on the size of the contact area. However, for static friction where there is an element of adhesion, the contact area does matter. Figure 3.5 below depicts the two forces of friction in terms of force against distance travelled, static friction is usually higher than dynamic friction, static friction is affected by area of contact between two objects.



Figure 3.5. A typical friction measurement output indicating difference between:  $\mu_S$  ("start-up" or "static") and  $\mu_D$  ("dynamic" or "sliding") friction.

The force of friction is always exerted in a direction that opposes movement. Rougher surfaces tend to have higher values. A value of zero would mean there is no friction at all. Static friction (informally known as stiction) occurs when the two objects are not moving relative to each other. The static coefficient of friction is typically denoted as  $\mu_s$ . The initial force to get an object moving is often dominated by static friction. Dynamic friction occurs when two objects are moving relative to each other and rub together. The coefficient of dynamic friction is typically denoted as  $\mu_D$ , and is usually less than the coefficient of static friction. From the mathematical point of view, however, the difference between static and dynamic friction is of minor importance. Let us have a coefficient of friction, which depends on the displacement velocity and is such that its value at 0 (the static friction  $\mu_s$ ) is the limit of the dynamic friction  $\mu_D$  for the velocity tending to zero.

### **3.4.2 Lubricants**

A common way to reduce friction is by using a lubricant, such as oil, that is placed between the two surfaces, often dramatically lessening the coefficient of friction. The science of friction and lubrication is called tribology. Lubricant technology is when lubricants are mixed with the application of science, especially to industrial or commercial objectives. "Tribology" is a term derived from the Greek word "tribos" meaning to rub, is defined as the science and technology of interacting surfaces in relative motion. The main subjects of study being, lubrication, friction and wear. A second term, "Biotribology" was defined in the 1970s as the study of all aspects of the subject of tribology related to biological systems, so it is wide ranging. Biotribology is the study of the tribology in biological systems e.g., teeth, hair, skin, eye and synovial joints (the interest of this study). The study of biotribology involves measurement of the resistance to motion of contacting surfaces expressed in terms of the coefficient of friction, which is generally symbolised by the Greek letter  $\mu$ . Figure 3.6 below, presents a close up picture of the Aston CSM Nano Scratch Bio-tribometer set up with a vibration free table allowing low coefficients of friction to be measured. Contact lenses are dehydrated for 5 minutes and fitted onto the mould as shown in Figure 3.6. A 100 $\mu$ l quantity of the solution was placed on the tribometer table (directly under the fitted contact lens). The friction force of the sliding contact lens against the solution under study was recorded as a function of distance travelled in mm ( $\sim$  0-20mm). A load of 60Mn (normal force) at a speed of 30mm/min was utilised for all frictional measurements carried out.

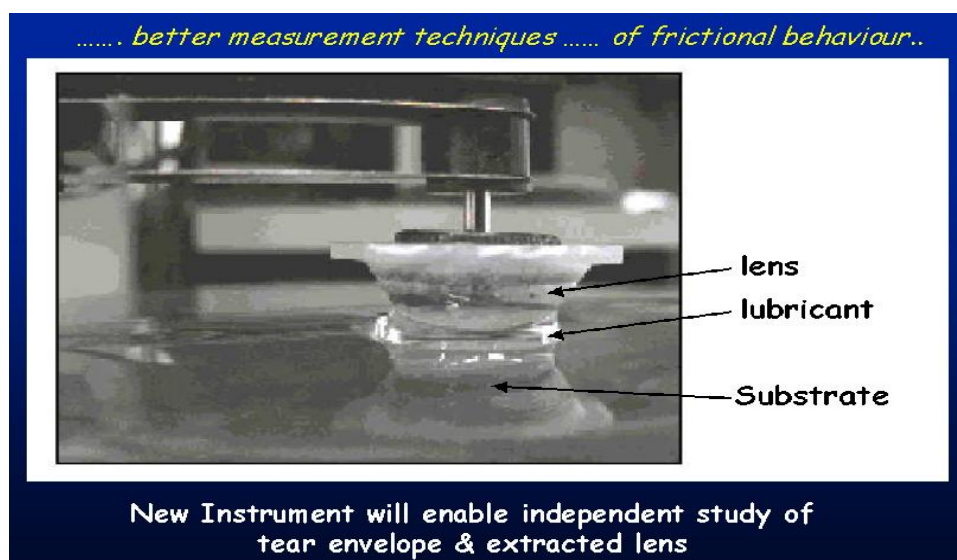


Figure 3.6. A close up view of the lens, lubricant and substrate.

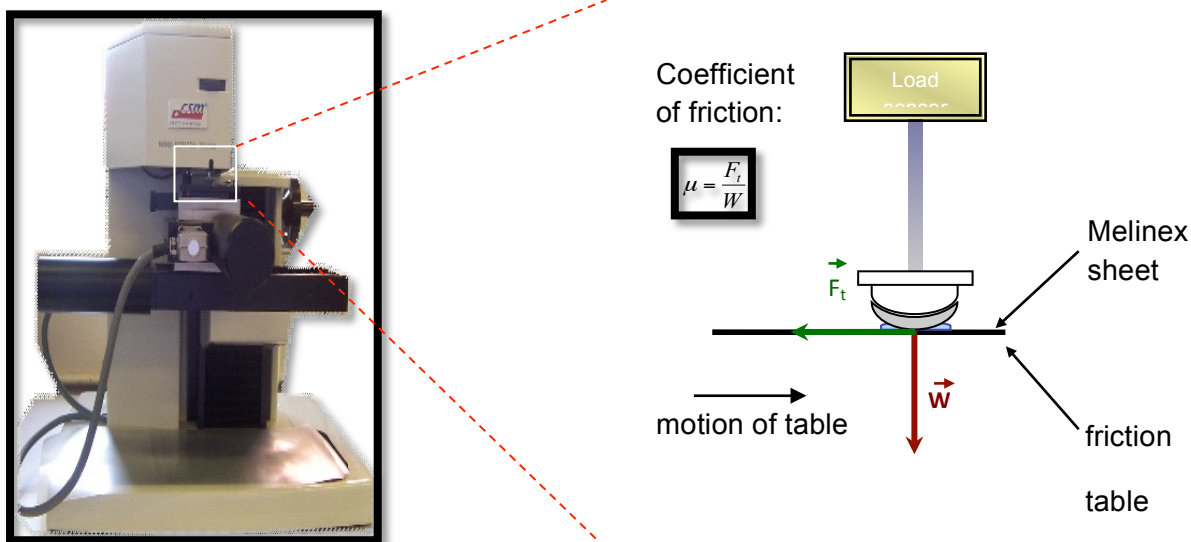


Figure 3.7. Aston CSM Nano Scratch Bio-tribometer set up with vibration free table allowing low coefficients of friction to be measured [87].

### 3.5 Osmometry; measuring osmolality

A osmometer is a device for measuring the osmotic strength of a solution, colloid, or compound. There are several different techniques employed in osmometry:

- Vapor pressure depression osmometers determine the concentration of osmotically active particles that reduce the vapour pressure of a solution.
- Membrane osmometers measure the osmotic pressure of a solution separated from pure solvent by a semipermeable membrane.
- Freezing point depression osmometer may also be used to determine the osmotic strength of a solution, as osmotically active compounds depress the freezing point of a solution.

Osmometers are useful for determining the concentration of dissolved salts or sugars in blood or urine samples. Osmometry is also useful in determining the molecular weight of unknown compounds and polymers. Osmometry is the measurement of the osmotic strength of a substance.

The osmolality of the solutions in this thesis was measured using a Camlab osmometer, this uses the freezing point depression principle effect. The osmole (Osm) is a non-SI unit of measurement that defines the number of moles of a chemical compound that contribute to a solutions osmotic pressure. Osmolarity is a measure of the osmoles of solute per litre of solution, while the osmolality is a measure of the osmoles of solute per kilogram of solvent. Molarity and osmolarity

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are not commonly used in osmometry because they are temperature dependent as a result of water changing its volume with temperature. However, if the concentration is very low osmolarity and osmolality are considered equivalent. In calculations for these two measurements, salts are presumed to dissociate into their component ions. For example, a mole of glucose in solution is one osmole, whereas a mole of sodium chloride in solution is two osmoles (one mole of sodium and one mole of chloride). Both sodium and chloride ions affect the osmotic pressure of the solution. The equation to determine the osmolality of a solution is given by :

$$\text{Osmolality} = \Phi nC \qquad \text{Equ 6}$$

Where;  $\Phi$  is the osmotic coefficient and accounts for the degree of dissociation of the solute.  $\Phi$  is between 0 and 1 where 1 indicates 100% dissociation.  $n$  is the number of moieties into which a molecule dissociates. For example:  $n = 1$  for glucose and 2 for NaCl,  $C$  is the molal concentration of the solution. The units are Osm/kg. Osmolality can be measured using an osmometer, which uses the principle of freezing-point depression to determine the osmolality. The depression of the freezing point in comparison to pure water is a direct measurement of osmotic concentration. In this thesis, the osmometer was used to measure the osmolality for some lubricants (under interest) in chapter 7.

#### **3.6 pH measurements**

The pH was measured with an Accumet® ABIO basic pH electrode. The pH meter was calibrated using pH 4, 7 and 10 buffer solutions, respectively.

#### **3.7 Freeze drying**

In the preparation of pharmaceutical formulations, drying is usually the final stage of processing and is designed to yield a stable homogenous product, which is easy to manipulate in subsequent operations of packing or formulating. The removal of water vapour from frozen solution by sublimation forms the basis of freeze drying. The process of drying from the frozen state is carried out by subjecting the material to be dried to low absolute pressure (high vacuum) after it has been frozen at temperature below  $-4^{\circ}\text{C}$ . Under these conditions, the frozen water will be sublimated. The water vapour is removed from the system by condensation in a cold trap maintained at a lower temperature than the frozen material. In general, moisture

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levels of freeze dried products are designed to be less than 3%. The Aston Medicines Research Unit (School of Life and Health Sciences), kindly allowed the usage of both their freezer and freeze dryer (Virtis Advantage, BioPharma Process systems, UK), for all the freeze drying carried out in this thesis. Firstly, the sample was dispensed in glass vials and frozen by maintaining the sample at  $-40^{\circ}\text{C}$  for 20 min. The frozen sample was immediately placed on the freeze-dryer plate at a temperature of  $-60^{\circ}\text{C}$ . Sublimation lasted 48 hours at a vacuum pressure of 10-13Pa and without heating, maintained at the condenser surface temperature of  $-60^{\circ}\text{C}$ . Finally, the glass vial was sealed under anhydrous conditions and stored at  $4^{\circ}\text{C}$  until being re-hydrated by using the same initial volume of deionised water [88].

#### **3.8 Measuring surface tension**

Surface tension is typically measure in units of dynes/cm, the force in dynes to break up a film length of 1cm. Equivalently it can be stated as surface energy in  $\text{ergs}/\text{cm}^2$  or by  $\text{mN}/\text{m}$ . There is a force of attraction between molecules in liquids and liquid can flow until the molecules can take on a shape that maximises the force of attractions. At the surface of the liquid, the force of cohesion between, the molecules is same, in all directions (see Figure 3.8). Molecules on the surface of the liquid, however, feel a net force of attraction that pulls them back in the body of the liquid. As a result the liquid tries to take on a shape that has the smallest possible surface area shape of a sphere. The magnitude of the force that controls the shape of the liquid is the surface tension [89].



Figure 3.8. Surface forces acting in a liquid, at a liquid- air interface [89].

##### **3.8.1 Measuring static surface tension via du Noüy ring method [89]**

The du Noüy ring (platinum-iridium, Pt-Ir) method is commonly used to determine the static surface tension of a liquid vapour or the interfacial tension of a liquid-liquid

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interface. The method of du Noüy ring is pulled through the liquid/vapour interface and the maximum downward force directed to the Pt-Ir ring is measured. As the maximum force and the parameters of the ring are known the surface tension can be calculated [90-92].

$$\gamma = \beta F / 4R \quad \text{Equ 7}$$

Where  $F$  is the pull on the ring,  $R$  is the mean radius of the ring and  $\beta$  is a correction factor. To ensure zero contact angle and, hence, a constant contact angle, platinum rings are carefully cleaned by flaming. It is essential the ring must lie flat in a quiescent surface. The correction factor  $\beta$  allows for the non-vertical direction of the tension forces and for the complex shape of the liquid supported by the ring at the point of detachment; hence, it depends on the dimensions of the ring and the nature of the interface.

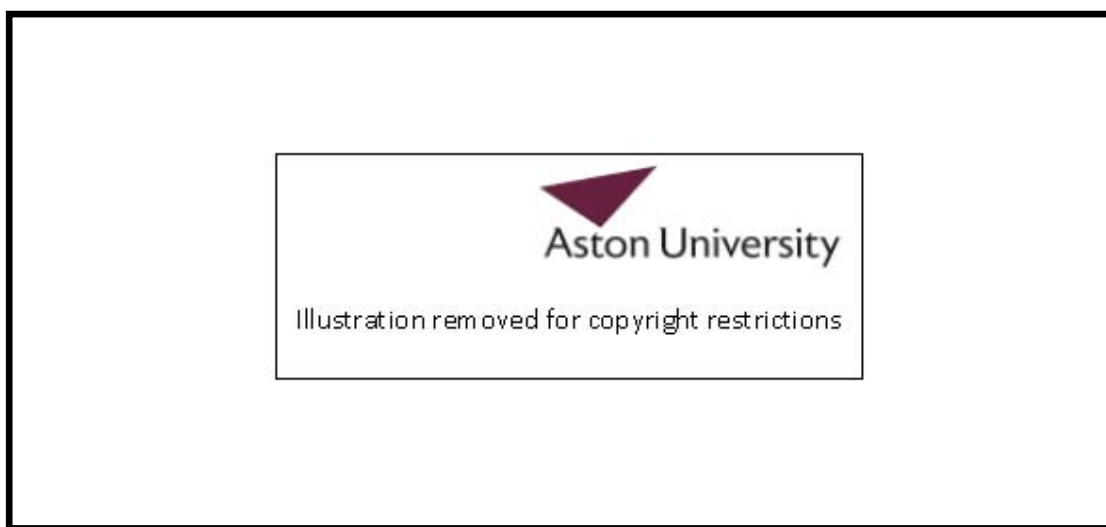


Figure 3.9. The du Noüy ring method [89].

The static surface tension of the solutions in this study were measured using a du-Noüy ring. All measurements were recorded to the nearest 0.1mN/m. The static surface tension was recorded at least three times for each sample. The mean and standard deviation for the results obtained were calculated in order to assess the viability of the data obtained.

#### **3.8.2 Measuring surface tension via the Wilhelmy plate/Langmuir method**

The easiest way possible to demonstrate the force arising from the surface tension is to dip a flat plate through the surface of a liquid and measure the force acting on it. . If the plate is perfectly wetted by the liquid a meniscus will form where it passes

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through the liquid surface giving a contact angle of  $0^\circ$ . If the plate is hanging vertically, the meniscus will contact the plate along a line of length  $2(x + y)$ , where  $x$  and  $y$  are respectively the horizontal length and thickness of the plate. Along the line of contact the liquid surface will be vertical so the surface tension along the line will, exert a downward force on the plate:

$$F = \gamma 2(x + y) \quad \text{Equ 8}$$

As  $F$ ,  $x$ , and  $y$  can all be measured, the surface tension can be determined. Thus the only correction that is needed arises from the buoyancy of the plate and that depends on the depth of the immersion. If, however, the bottom edge is set level with the flat surface of the liquid the buoyancy correction is zero. In the past, Wilhelmy plates have been made from a variety of materials, the most common being roughened mica, etched glass, and platinum. Scrupulous and elaborate procedures were needed with these materials to ensure that the surfaces were clean and that contact angle was zero. In 1977 Gaines suggested the use of paper plates. With high quality filter or chromatography paper the liquid saturates the plate and essentially forms a liquid surface over the paper ensuring that the contact angle is zero. In this thesis, work paper Wilhelmy plates were utilised during Langmuir studies [90-92].



Figure 3.10. The Wilhelmy plate [90].

In this thesis the dynamic surface tension was measured, with the use of a Wilhelmy plate balance, via the Langmuir trough technique. Wilhelmy plates made from paper are favoured as when they become saturated with water, receding and advancing angles are always zero. This allows reliable surface pressure to be measured in either compressed (receding contact angle) or expanding (advancing contact angle),

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and gives better control of surface pressure when it is being held constant for a measurement or a procedure.

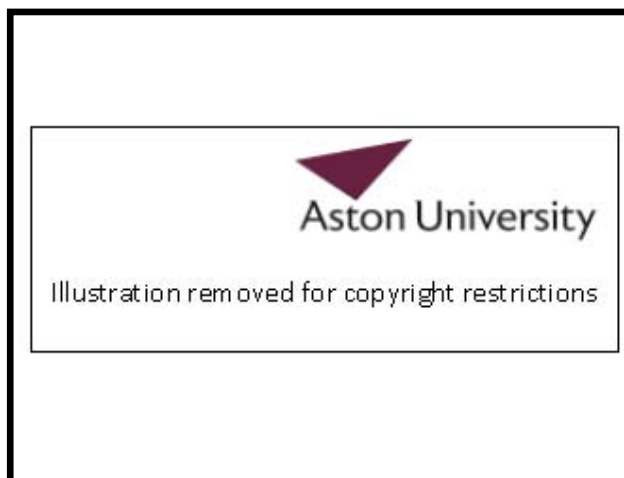


Figure 3.11. The Wilhelmy balance [92].

The surface tension can be defined, as 'the work required expanding the surface isothermally by unit area'. The tendency of surface active molecules to accumulate at interfaces favours expansion of the interface and hence lowers the surface tension. Such behaviour makes it possible to monitor the surface pressure as a function of the area occupied per molecule provided that the number of molecules deposited on the surface is known. The characteristics of a monolayer on the water surface are studied by measuring the changes in surface tension upon compressing the monolayer [92].

#### **3.9 Surface pressure and surface activity**

The association behaviour of surfactants in solution and their affinity for interfaces is determined by the physical and chemical properties of the hydrophilic and hydrophobic groups, respectively. The size and shape of the hydrophobic moiety and the size, charge and hydration of the hydrophilic head group are of utmost importance in this respect. The driving force behind the association is the reduction of the free energy of the system. Therefore, when a surfactant comes in contact with water it accumulates at the air-water interface causing a decrease in the surface tension of water. The strong adsorption at interfaces in the form of an orientated monomolecular layer (monolayer) is termed surface activity. Surface active materials (surfactants) consist of molecules containing both polar and non-polar parts (amphiphilic). Surface activity is a dynamic phenomenon, since the tendency towards adsorption and the tendency towards complete mixing due to the thermal



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motion of the molecules. The longer the hydrocarbon chain, the greater the tendency for molecules to adsorb at the air-water surface and, hence, lower the surface tension [93-95]. A rough generalisation, known as the Traube's rule, is that for a particular homologous series of surfactants the concentration required for equal lowering of surface tension in dilute solution decreases by a factor of about 3 for each additional CH<sub>2</sub> group[92]. Adsorption of self assembling films onto a water subphase retards evaporation. An example of this phenomenon in the biological environment is the lipoidal layer at the ocular surface. The tendency for surface active molecules to pack into an interface favours the expansion of the interface to contract under normal surface tension forces. If  $\pi$  is the expanding pressure (or surface pressure) of an adsorbed layer of surfactant, then the surface (or interfacial) tension will be lowered to a value of [90].

$$\gamma = \gamma_0 - \pi \quad \text{Equ 9}$$

#### 3.9.1 Langmuir theory

The plot of surface pressure versus the area of water surface available (at a constant temperature) to each molecule is known as the 'pressure-area' isotherm ( $\pi$  - A isotherm). The shape of the isotherm is characteristic of the molecules making up the film and hence provides a two-dimensional fingerprint. Phase transitions (gaseous, liquid expanded, liquid condensed and solid and eventually collapse), can be seen in a Langmuir isotherm, as films are compressed and expanded by the mechanical action of trough barriers [90-92]



Figure 3.12. Monolayer phase transitions [92].

The increase in surface pressure with compression of the surfactant film results from surface active molecules being forcibly inserted and crowded into the surface. Eventually there comes a point where the molecules slip over each other, and the film breaks and the monolayer is said to collapse at the maximum surface pressure.

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The collapse pressure ( $\pi_c$ ) is the maximum surface pressure reached when the film is compressed (squeezed) to a set minimum area. Here the film is displaced out of the surface. The higher the collapse pressure attained the more stable the bio-film is at the air-aqueous interface. On compression of the monolayer, some ordering of the film takes place and it behaves as would be expected of a two-dimensional liquid. With continued closing of the barrier, the increase in pressure causes additional ordering, the monolayer behaving as a quasi-solid. This solid state is characterised by a steep and usually linear relationship between surface pressure and molecular area. Eventually the collapse pressure  $\pi_c$  is reached at which the film irretrievably loses its mono-molecular form. The forces exerted upon it become too strong for confinement in two dimensions and molecules are ejected out of the monolayer plane into either the subphase (more hydrophilic molecules) or the superphase (more hydrophobic molecules) [90-92].



Figure 3.13. Collapse pressure [92].

The use of a Langmuir trough allows the measurement of surface pressure (reduction in surface tension) versus surface area isotherms (Langmuir isotherms) of liquids using only microlitre quantities, at the air-aqueous interface. When a monolayer is compressed on the water surface, the instantaneous surface tension ( $ST_{inst}$ ) of that surface is reduced - this reduction being known as the surface pressure (SP or  $\pi$ ). ST and SP have the same units (mN/m) and magnitude, but SP increases as the ST decreases: Hence:

$$ST_{inst} + SP = \text{Constant} \quad \text{Equ 10}$$

Constant = absolute ST of the liquid = 72.8mN/m for water at STP (298K, 1 atmosphere).



Figure 3.14. A stearic acid isotherm on pure water [90].

Surfactants are molecules, which are amphiphilic that is they are molecules, which are composed of a hydrophilic part and a hydrophobic part. Hydrophilic groups consist of groups such as carboxylic acid, sulphates, amines and alcohols. These are all attracted to polar media such as water and the forces acting upon them are predominantly coulomb type ( $1/r^2$ ). Hydrophobic (or oleophilic) groups such as a hydrocarbon chain, fats and lipids are much less (if at all) water soluble and the forces acting upon them are predominantly van der Waal's type ( $1/r^{12}$  and  $1/r^6$ ). Amphiphilic molecules are trapped at the interface because they possess these two very different types of bonding within the one molecular structure. When surfactants, dissolved in a non-aqueous volatile solvent, are introduced onto a polar liquid surface, the solvent will evaporate leaving the surfactants oriented at the liquid-gas interface. The hydrophilic 'head' groups pull the molecule into the bulk of the water and the hydrophobic 'tail' groups point into the air.

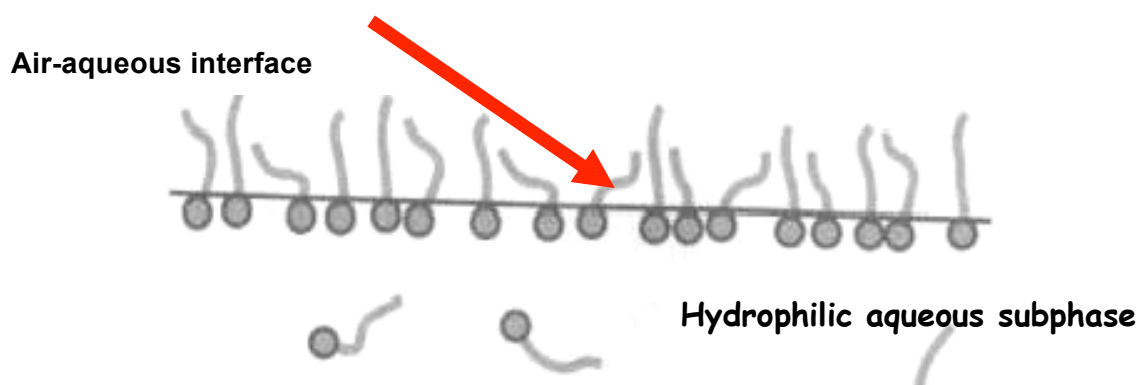


Figure 3.15. Orientation of molecules at air-aqueous interface,

A surface monolayer will only be achieved if the amphipathic balance of the molecule is correct; that is the balance between hydrophobic and hydrophilic parts. If the hydrophobic 'tail' group is too short (not hydrophobic enough) the molecule will be dragged into the water and will dissolve while if there is no hydrophilic part, the molecules may form thicker multilayer films on the surface or even evaporate. Sweeping a barrier over the water surface causes the molecules to come closer together and eventually to form a compressed, ordered monolayer. The film produced by such a method is known as a Langmuir film. Hence the interactions in the subphase are of longer range than those in the superphase. The isotherm can usually be seen to consist of three distinct regions. After initial spreading onto the subphase, no external pressure is applied to the monolayer and the molecules behave as a two-dimensional gas, which can be described by:

$$\pi A = kT \quad \text{Equ 11}$$

where  $\pi$  is the surface pressure,  $A$  the molecular area,  $k$  the Boltzmann constant and  $T$  is the thermodynamic temperature. However, collapse is not uniform across the monolayer but is usually initiated near the leading edge of the barrier or at discontinuities in the trough - such as corners or the Wilhelmy plate. Information can be obtained as to the way in which the molecules pack at the interface and the stability of the compressed layer at high pressures [90].

### 3.9.2 Cleaning the trough

The trough is fabricated with polytetrafluoroethylene (PTFE), a material which will not contaminate the surface. The trough was cleaned thoroughly prior to obtaining all Langmuir isotherms. The trough was wiped using surfactant free Kimwipe tissues soaked in chloroform (handled with the use of powder free gloves). The cleaning solvent is left to evaporate, and the trough surface then cleaned with HPLC grade

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water. The trough is then filled with HPLC grade water until the water just brims the surface. Once the pressure sensor has been calibrated and the Wilhelmy plate set up, the aqueous subphase needs to be cleaned. As the barrier is being closed any rise in surface pressure is due to contaminants on the subphase surface. In this situation the barrier need to be closed to the minimum area and the subphase cleaned with the PTFE nozzle (aspirator) connected to the suction pump. When the subphase is clean, i.e., there is no rise in surface pressure during compression the barriers are opened fully, and the surface pressure is zeroed. Langmuir isotherms can then be collected. This can take up to several hours to achieve sometimes.

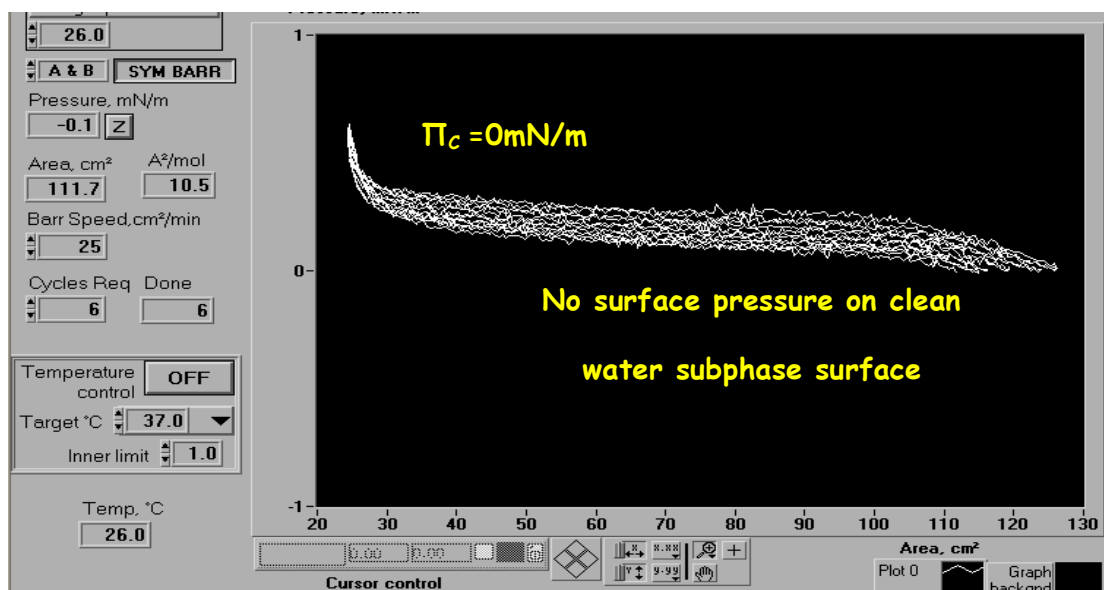


Figure 3.16. Subphase calibration - subphase cleaning.

#### 3.9.3 Pressure sensor calibration

Before each Langmuir isotherm is conducted the pressure sensor needs to be calibrated with 100mg calibration weights. An empty weighing pan is attached to the arm of the pressure sensor to zero the balance. A 100 mg weight is then attached to the pressure sensor, which reads a surface pressure of  $-46.7 \pm \text{mN/m}$ . Once the pressure sensor is calibrated, the Wilhelmy plate can be suspended from the pressure sensor arm.

#### 3.10 Brewster angle microscopy (BAM)

Brewster angle microscopy (BAM) allows the visualisation of surface Lipoidal film/microstructures at the air-water interface. A *p*-polarised light beam incident on

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the surface of water at the Brewster angle ( $53.4^\circ$ ) is not reflected. If a monolayer is spread on the surface it changes the refractive index and some reflection occurs. The reflected light can then be used to form a high contrast image of the lateral morphology of the spread or deposited layer. The BAM (BAM2 plus, Nanofilm Technologie, GmbH, Goettingen, Germany) was mounted above the Langmuir trough, to allow for collection of images of the morphology of monolayers and films. The Langmuir trough (monolayer prepared as described above) and the BAM were both housed in a cabinet to minimise disruption to the monolayer by air currents and to reduce airborne contamination. See Figure 3.18 for Aston experimental setup of a temperature controlled trough with BAM *P*-polarised light (supplied by an NDYAG laser operating at a wavelength of 532 nm) was emitted by the BAM onto the water surface at an angle of incidence of the Brewster angle for water and the light reflected from the surface collected by two achromatic lenses and detected with a charge coupled device (CCD) camera. The CCD camera converted the reflectivity signal from the sample into a video image. Calibrations were performed by obtaining images of the bare water surface prior to spreading the film. See Figure 3.19, for typical images obtained).

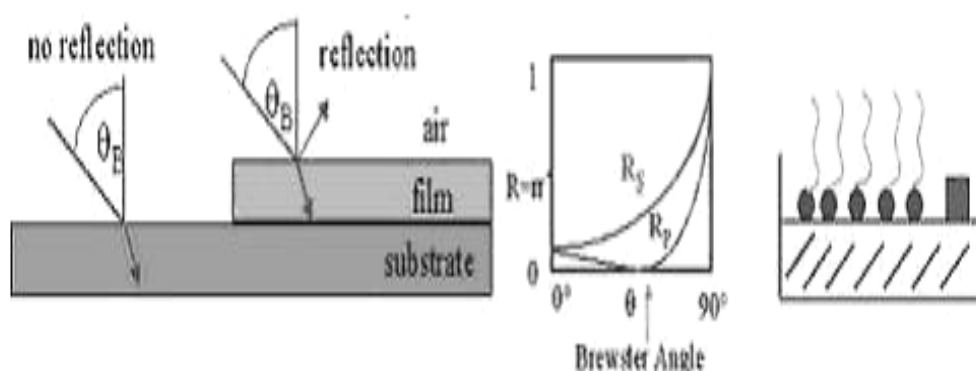


Figure 3.17. BAM principles.

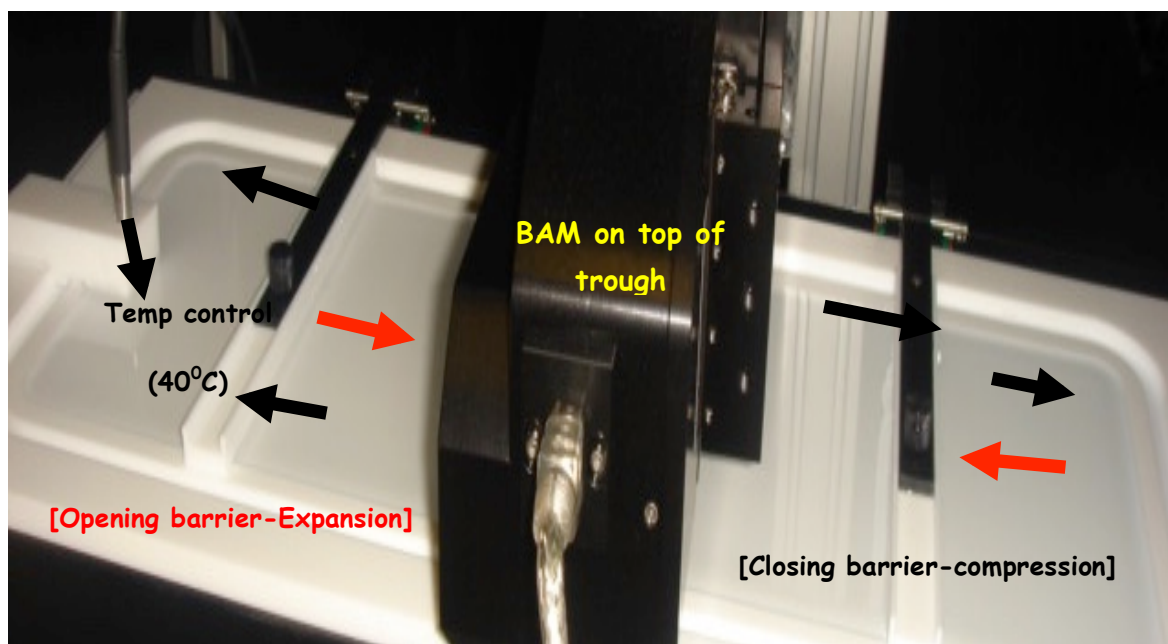


Figure 3.18. Aston experimental setup; a temperature controlled trough with a BAM.

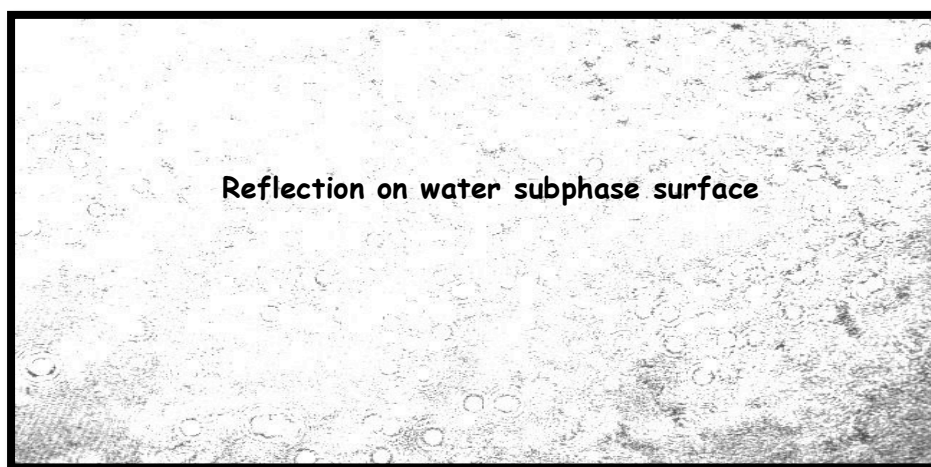
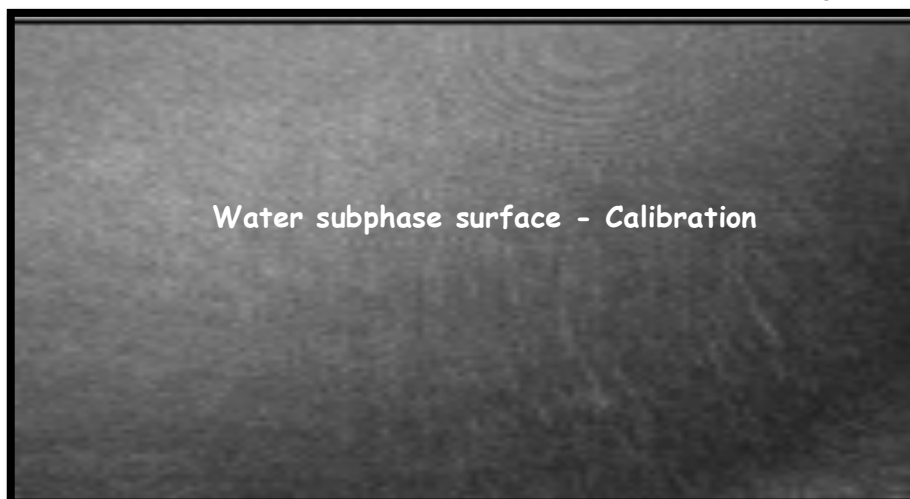


Figure 3.19. Typical microBAM images of the air-water interface (dark) and a deposited Lipoidal film (light).

**CHAPTER 4:**  
**THE STUDY OF POLY (STYRENE-CO-**  
**MALEIC ANHYDRIDE)-BASED**  
**COPOLYMERS BY NMR**  
**SPECTROSCOPY**



## **4.0 Introduction**

This Chapter focuses on studying PSMA-based copolymers via NMR spectroscopy. Initially the polymerisation synthesis routes for PSMA-based copolymers are investigated. A review has been conducted on a range of PSMA-based copolymers sought. NMR spectroscopy has played an important part in achieving the structure-property relationships, for the PSMA-based copolymers sought. The aim was to investigate PSMA-based copolymerisation mechanisms, polymer composition, monomer sequence distribution and stereostructure. Ultimately, it is important to understand the structure-property relationships for the development and the rational design of new functional smart PSMA-based materials.

Styrene and maleic anhydride have been believed to produce alternating copolymers that have been used in a variety of medical applications. In order to tailor the molecular engineering of PSMA-based copolymers for the application this thesis is concerned with, it is important to better our understanding of the complex chemistry associated with PSMA-based copolymers. The specific purpose of this work (Chapter 4, part 1) has been to seek, define and refine PSMA-based copolymer 'structures' with regards to developing effective lipid solubilising agents at the target physiological conditions, via NMR studies.

### **4.1 Free-radical copolymerisation of styrene and maleic anhydride**

Copolymerisation of styrene with maleic anhydride has been reported to yield alternating structures, probably due to the formation of charge transfer complexes. Statistical copolymers are produced if the process is carried out in a continuous batch reactor. Maleic anhydride itself does not homopolymerise and its copolymerisation with styrene has a strong tendency towards alternation, indicated by the reported reactivity ratio. The reactivity ratios  $r_1$  and  $r_2$  of styrene (monomer 1) and maleic anhydride (monomer 2) are 0.097 and 0.001 respectively, indicating that although both monomers preferentially react with the other, styrene is significantly less discriminating than maleic anhydride[93-97]. Consequently, the sequence distribution within a copolymer of styrene and maleic anhydride depends upon the monomer feed composition and the resulting copolymers can differ from 1:1 alternation. In the case where the ratio of styrene to maleic anhydride is greater than 1:1 (for example 2:1, 3:1 or 4:1) an increasing number of styrene-styrene sequences are present. This will result in a 'blocky' styrene monomer sequence arrangement.

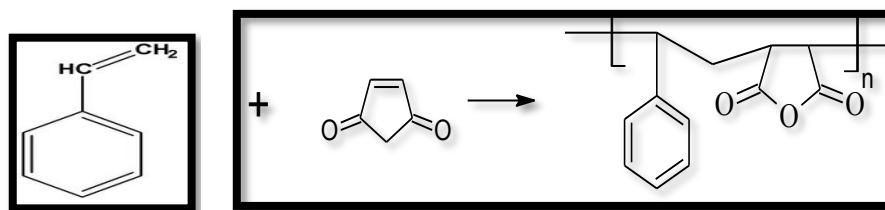


Figure 4.1. Polymerisation of styrene and maleic anhydride.

#### 4.1.1 Controlling molecular weight and microstructure

Batch mass polymerisation techniques have been commercially employed for preparing high molecular weight styrene-maleic anhydride copolymers. First, monomers are polymerised to an intermediate conversion. Slight modification of the maleic anhydride monomer by a certain alcohol usually is required to control the highly exothermic nature of the copolymerisation. The batch mass polymerisation technique can provide a high molecular weight styrene-maleic copolymer with a relatively high maleic content. All of the polymerisation reactions are carried out in dilute solutions using free-radical initiators such as peroxide or azo compounds (e.g., 2'-azobisisobutyronitrile or benzoyl peroxide). Extremely high molecular weight materials are difficult to obtain with the solution polymerisation process. Solution polymerisation is not presently employed on a commercial scale, due to the high cost involved. Most commercial processes for preparing styrene-maleic anhydride copolymers with high maleic anhydride contents are carried out in aromatic hydrocarbon solvents, including benzene, toluene, xylene, cumene and *p*-cymene. The molecular weight depends upon several factors including polymerisation temperature and solvent employed. Lower molecular weight polymers can be obtained from using aromatic solvents due to chain transfer functions of such solvents. By adding conventional chain transfers agents, it is possible to further decrease the molecular weight of the material. In addition, raising the polymerisation temperatures can decrease the molecular weight. Changing polymerisation process conditions can vary the polymer microstructure. If a large excess of styrene is charged, during the copolymerisation of styrene and maleic anhydride, a mixture of purely alternating styrene-maleic anhydride copolymers and homopolystyrene forms. If excess styrene content is desired into the polymer chain in a random fashion without homopolystyrene, a high polymerisation temperature is usually employed to break the donor/acceptor charge transfer styrene-maleic anhydride monomer complex. This approach leads to the polymer having a styrene 'blocky' structure, but the molecular weight is lowered

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due to the chain termination activity at high temperatures. This approach leads to the polymer having less styrene 'blocky' structure, but its molecular weight is lowered due to the chain termination activity at high temperatures [93-99].

### **4.1.2 Esterification of PSMA-based copolymers**

PSMA-based copolymers are very versatile; the polymeric materials can be modified. Much chemical modification work of the styrene-maleic anhydride copolymer has been done in order to change its degree of hydrophilicity and other properties. Examples include reactions with alcohols, ammonia, amines, and alkalies. Esterification of the styrene-maleic anhydride copolymer has been carried out by reacting the polymer with many different alcohols in organic solvents at high temperatures. The esterification reaction is usually stopped at the monoester stage, and a rigorous reaction condition is required to form diester polymeric product. Also, the esterification reaction of the styrene-maleic anhydride copolymer is reversible depending on reaction temperature. Usually a catalyst is required to facilitate the esterification reaction of the polymer [99]. The styrene-maleic half-ester copolymers are available commercially, usually in the form of methyl or butyl esters.

The poly[styrene-co-maleic acid/anhydride] half butyl ester is described in US4, 732, 933, as a pharmaceutical preparation conjugated to the antitumor agent neocarzinostatin, where the polymer acts to raise both the molecular weight and lipophilicity. The patent; JP01061424A, discloses a pharmaceutical formulation of SMANCS, a conjugate of a styrene/maleic acid mono butyl ester copolymer bound to molecule of the drug neocarzinostatin, prepared by mixing a solution of SMANCS in an ammonium carbonate buffer (pH 7.5-9.5) with a solution of a phospholipid such as egg yolk also in an ammonium carbonate buffer (pH7.5-9.5) to form a mixture which after being freeze dried to remove water is dispersed in a non-aqueous oily contrast medium to provide a clear and transparent dispersion therein of the SMANCS conjugate [57-62]. Styrene-maleic anhydride copolymers are hydrophobic and hydrophilic at the same time. The hydrophobic/hydrophilic nature of the polymer can be modified by adjusting the styrene and maleic anhydride ratio or by incorporating long hydrophobic alkyl chains or hydrophilic oxyalkene moieties. The materials are soluble in a number of different common organic solvents and can be solubilised into aqueous solution by converting the polymer into its inorganic or ammonium salts. The polymer film prepared with the ammonium salt of styrene-maleic anhydride copolymer esterified, with an alcohol of limited water solubility is almost insensitive to water. Variables

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introduced using these reactions include the type of alcohol used and the extent of the esterification reaction. The esterified resins can also be solubilised in water by reacting with excess base. In these reactions, the carboxylic acid groups of the partial esters are converted to their carboxylate salts, while anhydride groups in the resin react to give dicarboxylic acid salt functional groups. Therefore, the higher the anhydride content of the resin (or lower the styrene/maleic anhydride ratio), the higher the acid numbers of the resin, and the higher the solubility of the base hydrolysed product in water. Thus, between polymerisation chemistry and post reactions, varying systematic structural changes of styrene and maleic anhydride based copolymers can be achieved. In this work, we have attempted to understand what effect these variations have on structure-property relationships, in particular for the application this thesis is concerned with [93-99].

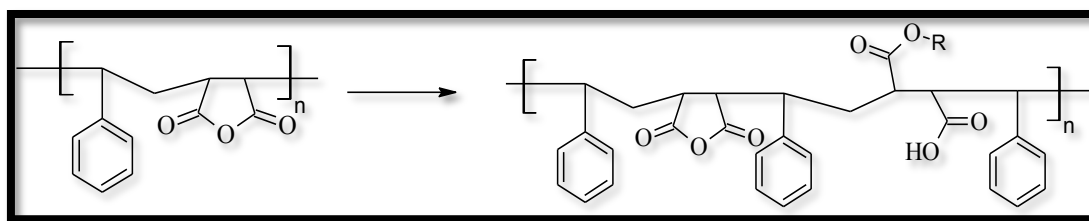


Figure 4.2. Partial esterification of PSMA-based copolymers.

Maeda and co-workers used low molecular weight PSMA copolymers (<6 kDa) clinically to deliver the antitumor protein neocarzinostatin (NCS). The polymer protein conjugate, known as SMANCS, is formed using 'partial half-esters' of SMA, in which 70% of the maleic anhydride groups were opened using butanol via an esterification reaction (see Figure 4.2 above for the reaction scheme for the partial esterification reaction of PSMA-based copolymers). SMANCS significantly improved the pharmacological properties of NCS by increasing both its circulatory half-life and its lipid solubility, and it has been clinically effective in treating liver cancer. The SMANCS conjugate is also known to accumulate in tumor tissue and the lymphatic system through the EPR effect (enhanced permeability and retention) [57-62].

### 4.1.3 Features of some commercially available PSMA-based copolymer

Copolymers of styrene and maleic anhydride have a long history. They come in a large variety of compositions and molar masses. The reactive maleic anhydride moiety provides the copolymers with a variety of options for chemical modification. The controlled synthesis and inherent post-polymerisation reactivity of PSMA-based copolymers make it an ideal starting material for complex architectures [96-101].

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Low molecular weight PSMA (MW = 1, 600, 1, 900, 9, 500, and 7, 500) copolymers were purchased from Polysciences (POL), with a styrene-to-maleic anhydride molar ratio of 1:1, 1: 2.3 and 3:1, respectively. Higher molecular weight PSMA-based (MW = 120, 000, 180,000 and 350,000) were purchased from Sigma-Aldrich (S-A). PSMA sodium salt solutions are available as a MW of 120, 000 and 350, 000 at 30% (wt/v) and 13% (wt/v) solutions. Modified PSMA-based copolymers, as mono-partial methyl ester (10-15%), propyl ester (50%), and isobutyl/mixed ester, were purchased from Sartomer (SAR) and S-A. SMA<sup>®</sup> resins from SAR are medium molecular weight PSMA-based copolymers. They are available as base polymers in varying styrene-to-maleic anhydride ratios (from 1:1 to 4:1 and beyond) and also as mono-partial monoesters. See Table 4.1 for some of the PSMA-based copolymers sought in this study.

PSMA-based copolymers come in a large variety of compositions and molar masses; see Table 4.1 for some features of the PSMA-based copolymers investigated in this work. The copolymerisation of styrene and maleic anhydride has received significant attention in academia as well as in industry. The underlying mechanism of the copolymerisation seems to still a point for debate. In recent years the copolymerisation of styrene and maleic anhydride has been reported in conjunction with living radical polymerisation techniques (nitroxide mediated polymerisation (NMP), and reversible addition fragmentation chain transfer (RAFT) mediated polymerisation [94-102]. NMR analysis has been conducted in order to investigate the PSMA-based copolymerisation mechanisms, polymer compositions, monomer sequence distributions and stereostructure. NMR analysis has also been utilised to provide evidence for the presence of various partial ester moieties reported by manufacturers, and to also examine the purity of the PSMA-based materials studied in this thesis.

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Table 4.1. Features of some commercially available PSMA-based copolymers studied.

<b>Code/MW</b>	<b>Features</b>	<b>Manufacturer</b>
NR1/1, 600	solid powder, full anhydride, 1:1	
NR2/1, 900	solid powder , full anhydride, 1:1	
NR3/9, 500	solid powder, full anhydride, 3:1	
NR4/7, 500	solid powder, full anhydride, 2:1	PO
NR5/120, 000	<u>30% wt/v, full anhydride, sodium salt solution, 1:1</u>	
NR6/350, 000	<i>mono-partial methyl ester (10-15%), solid powder, 1:1</i>	
NR7/1, 900	<i>mono-partial propyl ester (50%), solid, 1:2</i>	S-A
NR8/350, 000	<u>13% wt/v, sodium salt solution, 1:1</u>	
NR 9/180, 000	<i>mono-partial isobutyl/mixed ester, solid powder, 1:2</i>	
NR 10/5, 500	SMA <sup>®</sup> 1000P, solid powder, 1:1	
NR11/7, 500	SMA <sup>®</sup> 2000P, solid powder, 1:2	SAR
NR12/9, 500	SMA <sup>®</sup> 3000P, solid powder, 1:3	
NR13/7, 000	<i>SMA<sup>®</sup>1734, mono-partial methyl ester, solid powder, 1:1</i>	
NR14/5, 500	<u>SMA<sup>®</sup>1000HNA, sodium salt solution, 1:1</u>	

## **4.2 NMR Spectroscopy**

As is implied in the name, Nuclear Magnetic Resonance (NMR) is concerned with the magnetic properties of certain atomic nuclei. From proton NMR spectra we can deduce how many different kinds of hydrogen environments there are in the molecule, and also which hydrogen atoms are present on neighbouring carbon atoms. We can also measure how many hydrogen atoms are present in each of these environments. The object of the NMR experiment is to measure the frequency of a nuclear resonance. Studying an organic molecule by NMR spectroscopy enables us to record differences in the magnetic properties of the various nuclei present within the molecule. Signals appear on NMR spectrum corresponding to the different chemical and magnetic environments [73]. In this study, these principles were utilised to deduce the structures, microstructures and the stereostructure of the sought PSMA-based copolymers studied in this thesis. The nucleus of the hydrogen (proton) behaves as a tiny spinning bar magnet, and it does so because it possesses both electric charge and mechanical spin; any spinning charged body will generate a magnetic field, and the nucleus of hydrogen is no exception [73].

The NMR spectrometer accurately comprises of a strong, highly stable magnet in which the sample is placed and surrounded by transmitter/coils. Additional coils known as shims are also placed around the sample to counteract variations of field gradients spinning averages out inhomogeneities in the xy plane. By using these shims the field is made as homogeneous as possible. Remaining inhomogeneities are minimised by spinning the sample to counteract field gradients and render the field as perfectly homogeneous as possible. Spinning the sample tube about its axis means that the sample molecules experience average fields. Very well defined frequencies, and excellent resolution of close, narrow resonances, are obtained in this way. NMR is non-destructive, and with modern instruments good data may be obtained from samples weighing less than a milligram [73-85]. The difference in precessional frequency corresponds to a difference in chemical environment, as the shift in frequency is dependent on chemical environment, this gives rise to the term chemical environment. To measure the precessional frequency of a group of nuclei in an absolute frequency units is difficult and rarely required. More commonly the differences in frequency are measured with respect to some reference group of nuclei. For protons, the universally accepted reference is tetramethylsilane (TMS).

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Hydrogen nuclei are surrounded by electronic charge, which to some extent shields the nucleus from the influence of the applied magnetic field  $B_0$ , and to bring a proton to resonance, the magnetic flux must overcome this shielding effect. In a magnetic field, the electrons around the proton are induced to circulate, and in doing so they generate a small secondary magnetic field, which acts in opposition (that is diamagnetically) to the applied field. The greater the electron density circulating around the proton, the greater the induced diamagnetic effect, and the greater the external field required to overcome the shielding effect. Electronegativity groups withdraw electron density (inductive effective) and these deshielding effects mean that a lower value of the applied magnetic field is needed to bring the nuclei to resonance. The  $^1\text{H}$ -NMR spectra of most organic molecules contain proton signals that are 'split' into two or more sub-peaks. Rather than being a complication, however, this splitting behaviour actually provides us with more information about our sample molecule. The source of signal splitting is a phenomenon called spin-spin coupling, a term that describes the magnetic interactions between neighbouring, non-equivalent NMR-active nuclei. The spin-spin interaction of neighbouring hydrogens takes place through the covalent bonds that join them. The most common bonding relationship is vicinal (joined by three sigma bonds). In this case a neighbouring proton having a  $+1/2$  spin shifts the resonance frequency of the proton being observed to a slightly higher value (up to 7 Hz), and a  $-1/2$  neighbouring spin shifts it to a lower frequency. Remember that the total population of these two spin states is roughly equal, differing by only a few parts per million in a strong magnetic field. If several neighbouring spins are present, their effect is additive. The statistical distribution of spins within each set explains both the  $n+1$  rule and the relative intensities of the lines within a splitting pattern. The number of lines (multiplicity) observed in NMR signal for a group of protons is related to the number of protons in the group; the multiplicity of lines is related to the number of protons in the neighbouring groups. For example the NMR spectra molecule  $\text{CH}_3\text{CH}_2\text{Cl}$  you will see a quartet for the  $\text{CH}_2$  type protons and a triplet for the  $\text{CH}_3$  type protons respectively.

Rigid molecules occupy a sterically hindered position, and in consequence the electron cloud of the hindering group will tend to repel, by electrostatic repulsion, the electron cloud surrounding the proton. The proton will be deshielded and appear at a higher chemical shift. The chemical shift positions for protons attached to  $\text{C}=\text{C}$  in alkenes is higher than can be accounted for by electronegativity effects alone. The same is true for aldehydic protons and aromatic protons, whereas alkyne protons appear at relatively low chemical shift positions. The explanation is again collated with the manner in which electrons, in this case  $\pi$ -electron, circulate under the influence of the



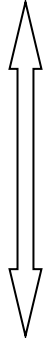
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applied field. The effect is complex, and can lead to downfield shifts (paramagnetic shifts) or upfield shifts (diamagnetic shifts). In addition, the effects are paramagnetic in certain directions around the  $\pi$  clouds, and diamagnetic in others, so that these effects are described as anisotropic, as apposed to isotropic (operating equally through space). When an alkene group is so orientated that the plane of the double bond is at  $90^\circ$  to the direction of the applied field induced circulation of the  $\pi$ -electrons generates a secondary magnetic field, which is diamagnetic around the carbon atoms, but paramagnetic (that is, it arguments  $B_0$ ) in the region of the alkene protons. Where the direction of the induced magnetic field is parallel to the applied field  $B_0$ , the net field is greater than  $B_0$ , protons in these zones require a lower value of  $B_0$  to come to resonance, and therefore appear at lower field (higher chemical shift values) than expected [73-85]. For the carbonyl group a similar situation arises, although the best representation of shielding and deshielding zones is slightly different from the alkene pattern. Two cone-shaped volumes, centred on the oxygen atom, lie parallel to the axis of the C=O bond; protons within these cones experience deshielding, so that aldehydic protons, and the formyl protons of formate esters appear at high chemical shift values. Protons held above or below these cones will come to resonance at lower chemical shift values. Whereas alkene and aldehydic protons appear at high chemical shift values, alkyne protons appear around 1.5-3.5  $\delta$ . Electron circulation around the triple bond occurs in such a way that the protons experience a diamagnetic shielding effect. When the alkyne group lies parallel to the direction of  $B_0$  in the vicinity of the protons, higher  $B_0$  is needed to bring the protons to resonance; therefore acetylenic protons appear at low chemical shift. In the molecule of benzene (and aromatic compounds in general)  $\pi$  electrons are delocalised cyclically over the aromatic ring. These loops of electrons are induced to circulate in the presence of the applied field  $B_0$ , producing a substantial electric current called the ring current. Anisotropic shielding and deshielding are associated with the aromatic ring current. The induced field is diamagnetic (opposing  $B_0$ ) in the centre of the ring, but the returning flux outside the ring is paramagnetic (augmenting  $B_0$ ). Protons around the periphery of the ring experience a magnetic field greater than  $B_0$  (higher  $\pi$  -  $\delta$   $\delta$  values) than would otherwise be so. Protons held above or below the plane of the ring resonate at low  $\delta$  values. Simple electronegative (inductive) effects operate only along a chain of atoms, the effect weakening with the distance, but magnetic anisotropy operates through space irrespective of whether the influenced group is directly joined to the anisotropic groups and are likely to have an influence on the chemical shift of apparently distant

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protons. Predicting the NMR spectrum of an organic compound begins with predicting the chemical shift positions, for the different hydrogens in the molecule [73-85].

Table 4.2. Approximate chemical shift positions for protons in organic molecules [73].

<b>Chemical shift <math>\delta</math> (ppm)</b>	<b>Functional group</b>	<b>Chemical environment</b>
1-2ppm	CH <sub>2</sub> CH <sub>3</sub>	 Shielded (lower $\delta$ ) - Upfield
2-3ppm	CH <sub>3</sub> N	
3-4ppm	CH <sub>3</sub> O	
2-3.4ppm	CH $\equiv$ C-H	
5-6.5ppm	Alkenes	
6.5-8ppm	Aromatics	
9-10ppm	CHO	
11-12ppm	COOH	
13+ppm	Enols	Deshielded (higher $\delta$ ) - Downfield

### 4.2.1 <sup>1</sup>H-NMR spectra analysis for PSMA-based copolymers

To satisfy the condition that the nonviscous samples give the sharpest NMR spectra, it is usually necessary to record the spectra in solution. A deuterated solvent is utilised so no peaks due to hydrogen can be observed, however the purity level is not always 100%, so peaks are sometimes present due to residual matter. The PSMA-based copolymer under study was dissolved in deuterated acetone (d<sub>6</sub>). The peaks that are due to the residuals in the deuterated solvent are visible at 2.04ppm (in the <sup>1</sup>H-NMR spectra) and at 29.8ppm, 206 ppm (for the <sup>13</sup>C-NMR spectra), respectively. 32 scans were collected for each sample on a 300MHz NMR spectrometer. The Fourier transform was processed using the XWIN-NMR 3.5 Bruker NMR software. Examination of the simplest theoretical alternating repeat unit of styrene maleic anhydride, predicts that there are four different hydrogen chemical environments.

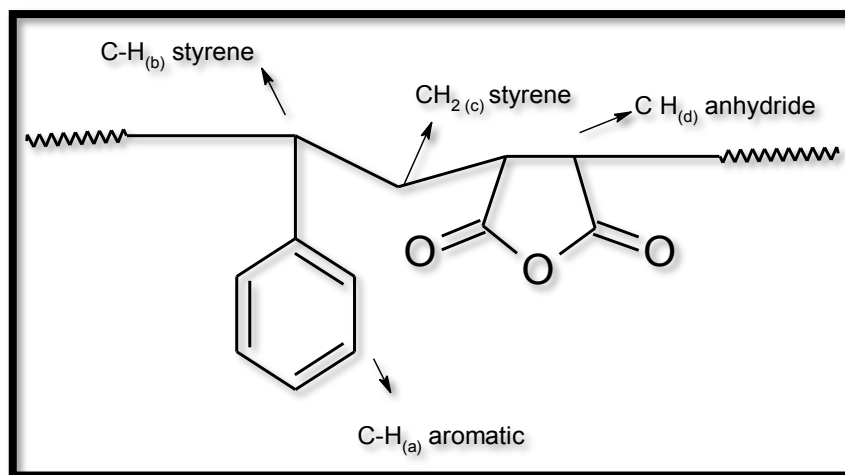


Figure 4.3. Types of proton chemical environments in a theoretically perfectly alternating PSMA repeat unit (full anhydride).

If the PSMA-based copolymer under study were perfectly alternating (as reported in the literature) we would only see four different  $^1\text{H}$ -NMR peaks. In order of deshielding (higher chemical shift), four different chemical environments would be identified; a-b (6.5-8ppm), d(~3ppm), c(~2ppm), respectively, see Figure 4.3 above for representation of these four different  $^1\text{H}$  nuclei chemical environments. The assignments have been assigned with reference to Table 4.2 and in accordance to all the factors that affect the chemical shift observed for the hydrogen nuclei under investigation. The areas under each signal are in the ratio of the number of protons in each part of the molecule, and measurement will show that the ratio of the area and has also been measured and included (e.g. see below in Table 4.3) in the NMR analysis for the PSMA-based copolymers studied here.

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Table 4.3. Typical  $^1\text{H}$ -NMR spectra peaks observed for PSMA, POL, MW 1, 600 (NR 1) based copolymer.

<b>Functional group</b>	<b>Chemical shift <math>\delta</math> ppm (integral)</b>	<b>Monomer component</b>	<b>Position (see Figure 4.3)</b>
$\text{CH}_3$ , methyl	1.3-1.6ppm	Impurities	not part of molecule
$\text{CH}_2$ , methylene (c)	2.1-2.7ppm	styrene	c
CH, methine (d)	3.1-3.7ppm (2.26)	maleic anhydride	d
residual styrene, C-H (b) styrene	6.5-6.9ppm	styrene	b
C-H aromatic (a) (para, ortho & meta)	7-7.8ppm (4.0)	styrene	a, b
solvent peaks	2.04ppm	deuterated acetone	

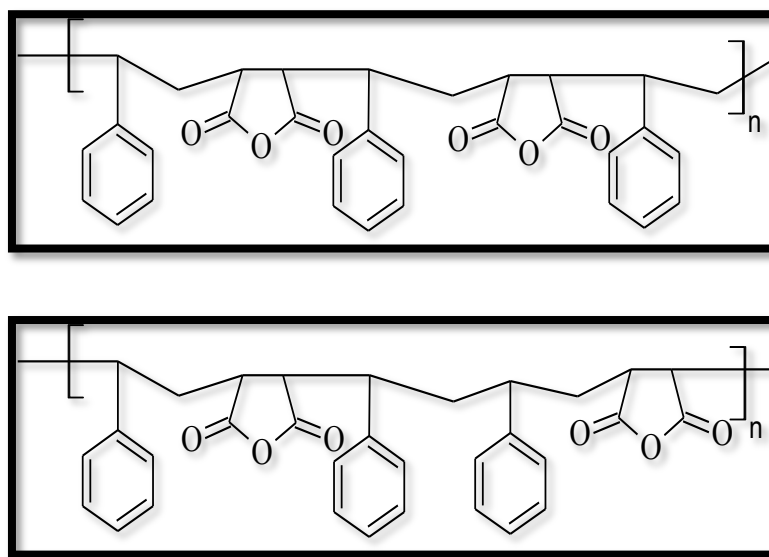


Figure 4.4. Some possible monomer sequences for PSMA POL, MW = 1,600 (NR 1).

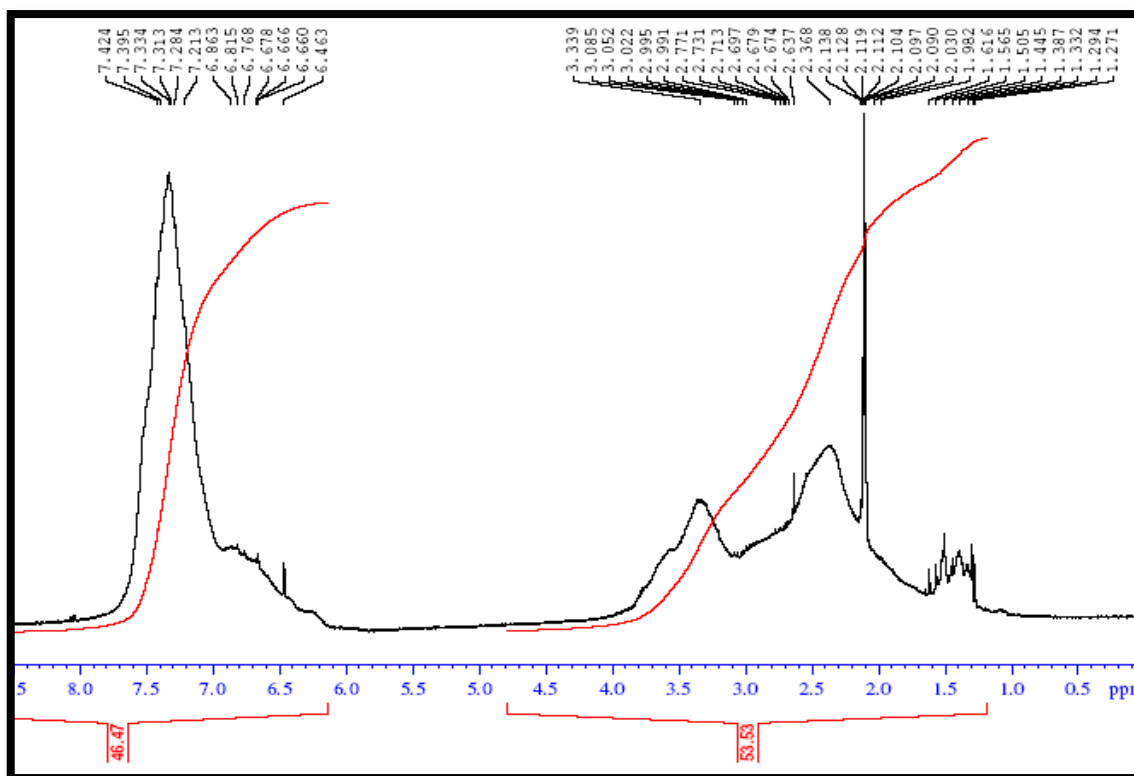


Figure 4.5. <sup>1</sup>H-NMR spectra of alternating PSMA (POL), MW = 1,600, (NR1), in deuterated acetone (d<sub>6</sub>).

47: 53 ~ 1:1.2

Figure 4.5 above, depicts that even the polysciences (MW 1, 600) 1:1 monomer feed ratio - NR1, PSMA-based copolymer, is actually not perfectly alternating, as more than four peaks are present in the generated spectra. If however, the polymer were perfectly alternating only four hydrogen chemical environments would have been visible as depicted in a Figure 4.3 for a theoretical alternating PSMA-based copolymer. The peaks are in fact very broad with ample peaks (illustrating the complexity of the microstructure of PSMA-based copolymers) overlapping indicative of polymer spectra, as expected. This fact makes the NMR analysis very challenging indeed. This strongly suggests a broad 'triad' monomer sequence distribution content, confirming a non-alternating monomer sequence distribution.

However, in contrary Scott M. Henry, *et al* [103] report that styrene and maleic anhydride produce alternating copolymers, which has also been reported by many other researchers too. Tzong-Liu Wang *et al* [104] present the <sup>1</sup>H-NMR for the SMA copolymer; the resonance peak at 7.2-7.4ppm is ascribed to the protons of the benzene ring, while the peak at 2.35ppm is associated with the protons of the maleic

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anhydride backbone [104]. These peak assignments are similar with what is revealed in this study, however, here we examine the NMR data obtained in much more detail.


Referring to Table 4.3, the characteristic peaks due to the aromatic protons can be found between  $\delta \sim 7\text{-}7.8\text{ppm}$ . These are labelled as 'a' in Figure 4.3. The slight variations in these 5 C-H aromatic are due to the ortho, meta, and para position in the styrene ring, respectively. The position of these hydrogens in styrene are indicated by numbers except that ortho, meta and para, may be used in place of 1,2 1,3 and 1,4 positions. The peaks at  $\delta \sim 6.5\text{ to }6.9\text{ppm}$ , are due to alkene styrene groups (residual styrene monomer). The aromatic protons are more deshielded and hence appear at higher chemical shift values (upfield). The peaks at  $\delta \sim 3.1\text{-}3.7\text{ppm}$  are for the CH, methine group (labelled 'd' in Figure 4.3) on the maleic anhydride functionality. The methine hydrogens are more shielded and hence appear at a lower chemical (downfield). The peaks at  $\delta = 2.1\text{-}2.7\text{ppm}$ , are ascribed as the styrene methylene functional group  $\text{-CH}_2\text{-}$ , labelled as 'c' in Figure 4.3. These methylene hydrogens are even more shielded and thus appear at an even lower chemical shift value. Dividing the anhydride peak ( $\delta \sim 3.1\text{-}3.7$ ) by 2 gives the ratio of maleic anhydride in the copolymer. Dividing the aromatics peak ( $\delta \sim 7\text{-}7.8\text{ ppm}$ ) by 5, gives the ratio of styrene in the copolymer. The ratio of the two indicates the approximate copolymer maleic anhydride-to-styrene monomer ratio;

- ( $\delta = 3.1\text{-}3.7\text{ppm}$ )  $\sim 2.26/2 = 0.75/0.75 = 1 \sim \text{MA}$  (see Figure 4.5 for data used)
- ( $\delta = 7\text{-}7.8\text{ ppm}$ )  $\sim 4/5 = 0.8/0.75 \sim 1.07 = \text{ST}$  (see Figure 4.5 for data used).

Therefore, the copolymer composition is approximately 1:1.2, for PSMA MW = 1,600 (NR1).

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Table 4.4.  $^1\text{H}$ -NMR spectra peaks assignments for PSMA SMA<sup>®</sup> 2000P (2:1), NR 11 based copolymer.

<b>Functional group</b>	<b>Chemical shift  (ppm)</b>	<b>Monomer component</b>	<b>Position (see Figure 4.3)</b>
CH <sub>3</sub> , methyl	1.3-1.4ppm	Impurities	not part of molecule
CH <sub>2</sub> , methylene (c)	2.1-2.6ppm, 2.9ppm	styrene	c
CH, methine (b)	3.0-3.6ppm	maleic anhydride	d
C-H aromatic (para, ortho and meta) (a)	6.6-7.7ppm	styrene (residual styrene monomer)	a , b
C-H aromatic (b), quaternary Hs	8ppm	styrene	a, b
solvent peaks	2.04ppm	deuterated acetone.	

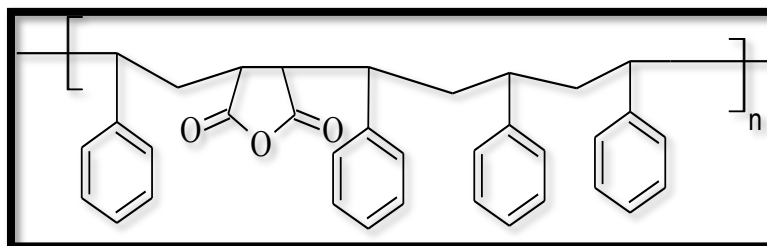


Figure 4.6. Possible monomer sequence distribution for blocky (non-alternating triad) PSMA-based copolymer.

Figure 4.7, shows that the,  $^1\text{H}$ -NMR spectra for SMA<sup>®</sup>2000P (2:1), NR11 demonstrates slightly shifted peaks for all four types of hydrogen, and more so with regards to a few magnetically inequivalent methylene hydrogen environments.

Figure 4.8, illustrates that the MW 7,500, SMA<sup>®</sup>3000P (3:1), NR12 spectra depicts slight variations associated with the aromatic region, as expected due to being a higher styrene content material.

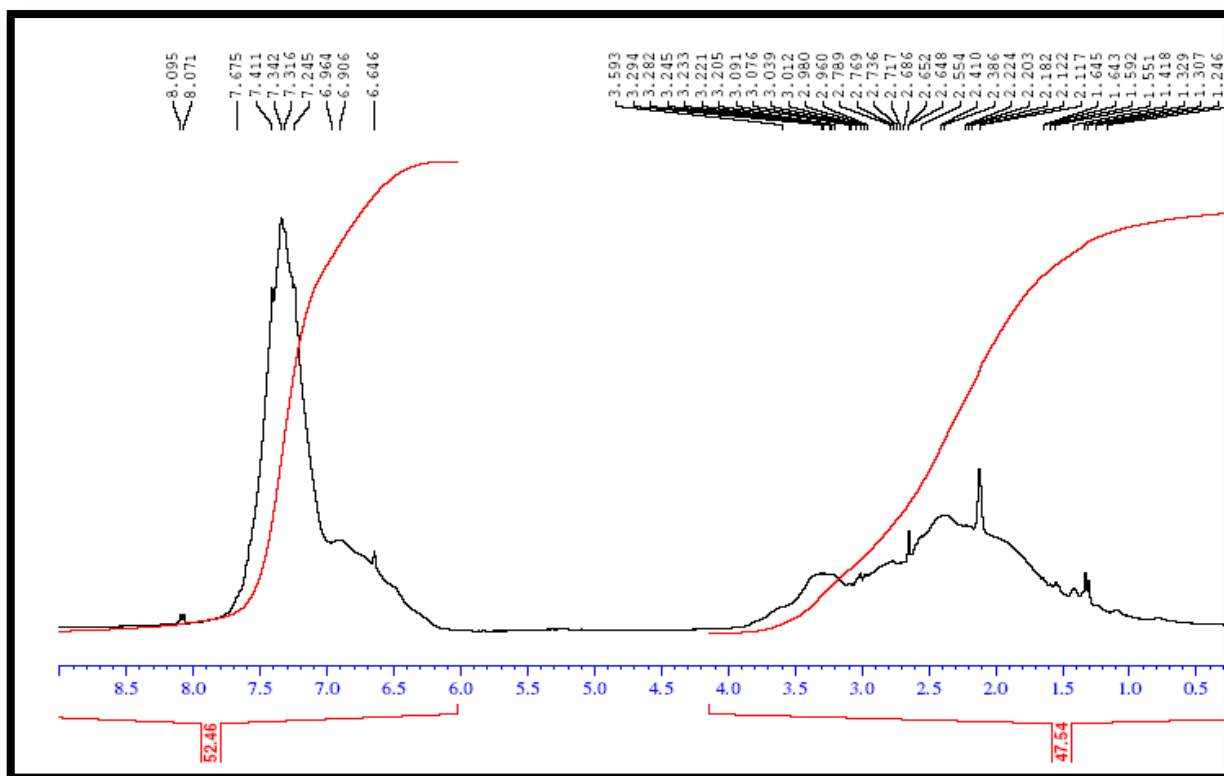


Figure 4.7.  $^1\text{H}$ -NMR spectra of blocky SMA<sup>®</sup>2000P(2:1) NR11, in deuterated acetone ( $\text{d}_6$ ).

(52 ; 47 ~ 1.11 : 1)

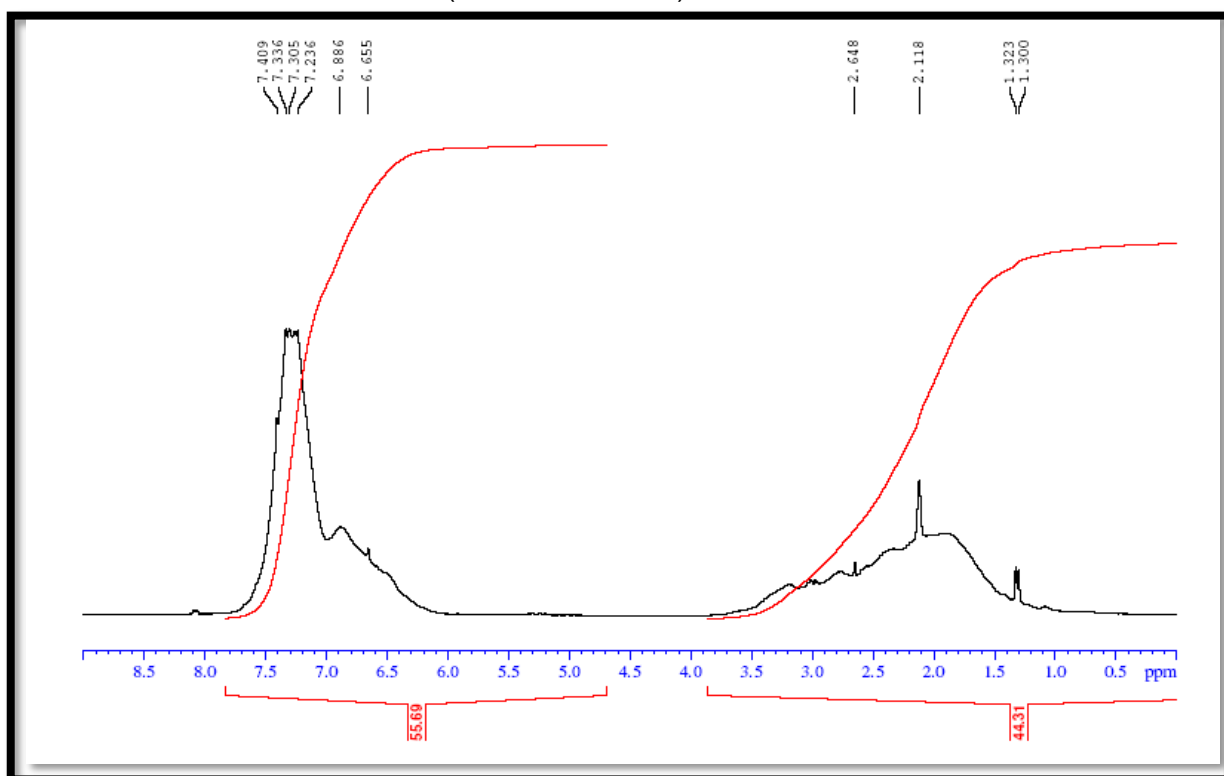


Figure 4.8.  $^1\text{H}$ -NMR spectra of blocky PSMA MW 7,500, SMA<sup>®</sup>3000P (3:1), NR12, in deuterated acetone ( $\text{d}_6$ ).

(56: 44 ~ 1.3: 1)



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Table 4.5.  $^1\text{H}$ -NMR spectra peaks assignments for PSMA SMA<sup>®</sup> 3000P based copolymer.

<b>Functional group</b>	<b>Chemical shift <math>\delta</math> (ppm)</b>	<b>Monomer component</b>	<b>Position (see Figure 4.3)</b>
$\text{CH}_3$ , methyl	1.4ppm	impurities	not part of molecule
$\text{CH}_2$ , methylene (c)	2.1-2.6ppm	styrene	c
CH, methine (d)	3.0-3.4ppm	maleic anhydride	d
C-H, styrene (a,b)	6.6-6.9 ppm	residual monomer	
C-H, aromatic (para, ortho and meta) (a,b)	7-7.7ppm	styrene	a, b
C-H, aromatic (b)	8ppm	styrene	a, b
solvent peaks	2.04ppm	deuterated acetone	

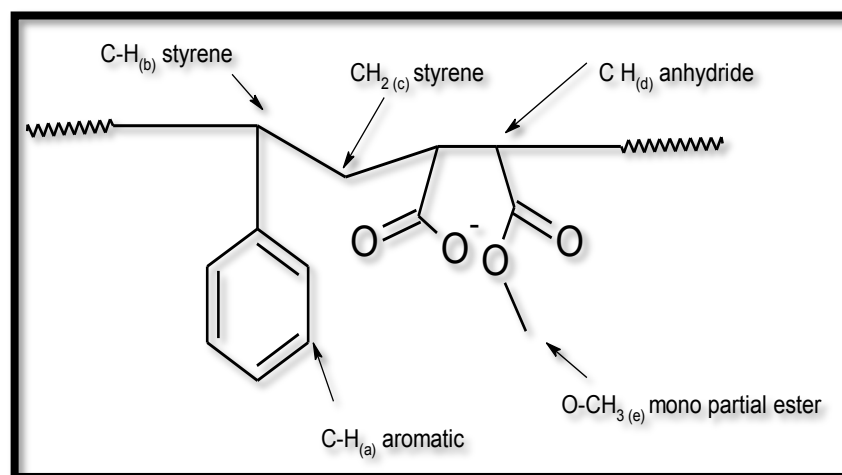


Figure 4.9. Additional types of protons in PSMA, mono-partial methyl ester. (please note structure shows charged PSMA, as it is a partial ester).

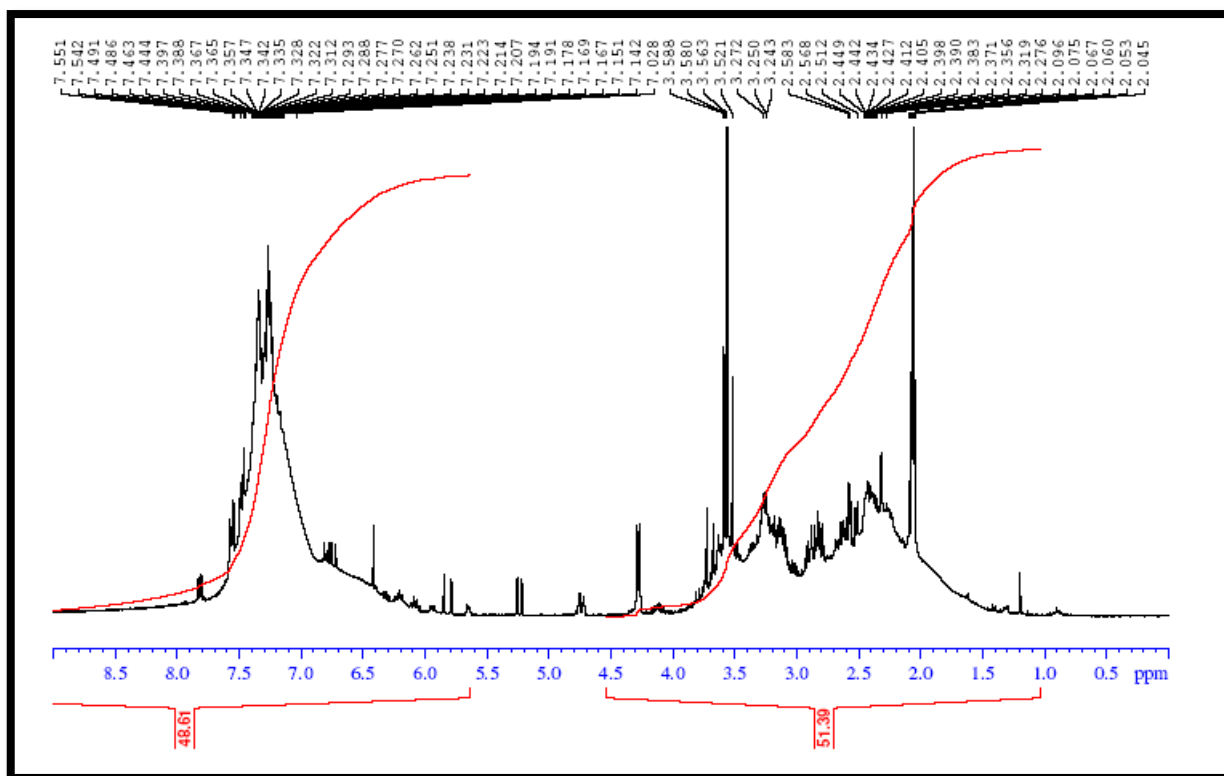


Figure 4.10.  $^1\text{H}$ -NMR spectra of alternating PSMA MW = 350, 000 - mono-partial methyl ester (10% wt/v), POL, NR6, in deuterated acetone ( $\text{d}_6$ ).

(49: 51 ~ 1: 1.04)

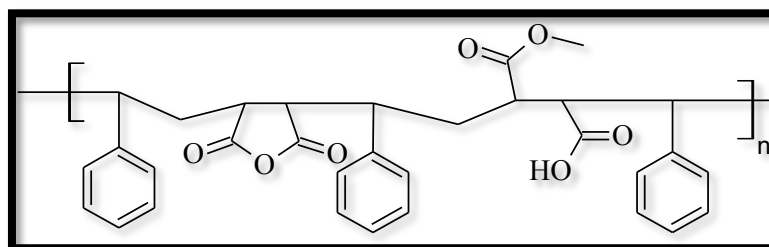


Figure 4.11. Possible monomer sequence distribution for alternating PSMA, mono-partial methyl ester based copolymers.

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Table 4.6. <sup>1</sup>H-NMR spectra peaks assignments for PSMA, mono-partial methyl ester (NR 6).

<b>Functional group</b>	<b>Chemical shift <math>\delta</math> (ppm)</b>	<b>Monomer component</b>	<b>Position (see Figure 4.9)</b>
CH <sub>3</sub> , methyl	1.4ppm	Impurities	not part of molecule
CH <sub>2</sub> , methylene (c)	2.1-2.9ppm	styrene	c
CH, methine (d)	3.0-3.4ppm	maleic anhydride	d
O-CH <sub>3</sub> (deshielded methyl, electronegativity effects) (e)	3.5-4ppm	mono partial methyl ester	e
impurities, unreacted monomer (a,b)	4.7-5.8ppm	styrene	
residual styrene monomer	6.4-6.7ppm	styrene monomer	styrene
	6.6-7.7ppm	styrene	b
C-H aromatic (a,b) (para, ortho and meta)			
C-H aromatic (a.b)	8ppm	styrene	a
solvent peaks	2.04ppm	deuterated acetone.	

Figure 4.10, provides evidence that PSMA MW = 350, 000 - mono-partial methyl ester (10% wt/v), POL, NR6, is in fact a partial ester. New peaks are apparent which are due to a methyl ester group, at  $\delta \sim 3.5\text{-}4\text{ppm}$ . These appear at a higher chemical shift value due to electronegativity (from the oxygen group) and electronic shielding effects.

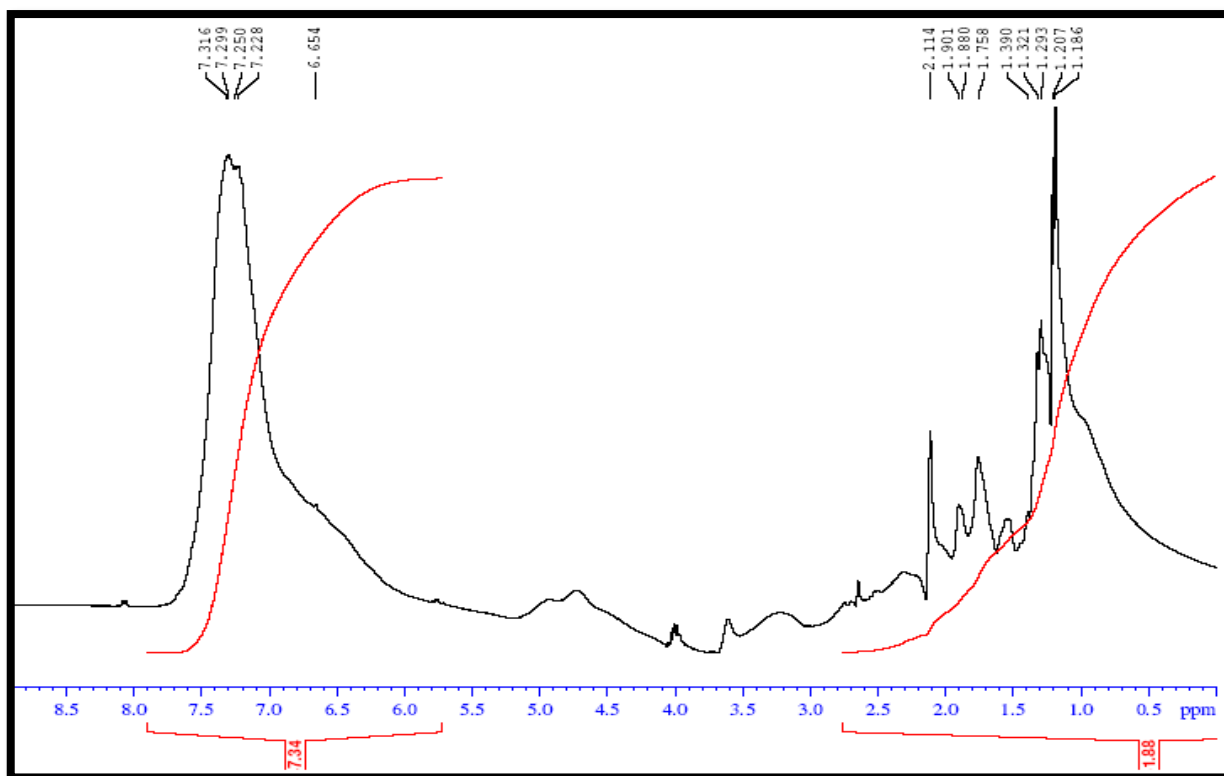


Figure 4.12.  $^1\text{H}$ -NMR spectra of alternating PSMA MW = 7,000 - mono-partial methyl ester (NR13) in deuterated acetone ( $\text{d}_6$ ).

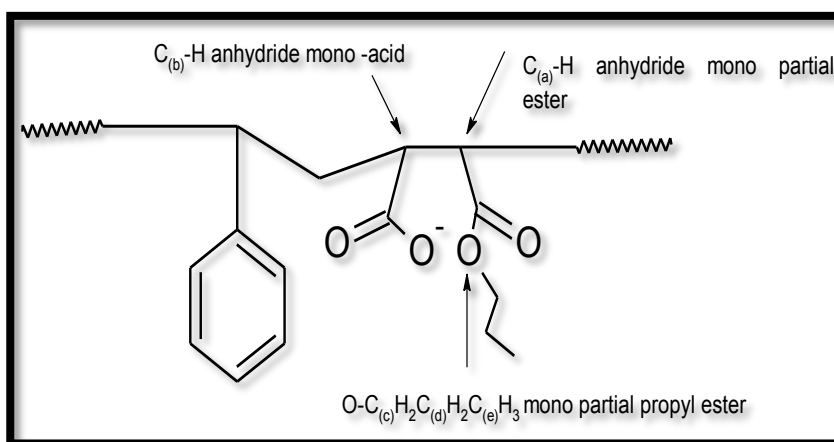


Figure 4.13. Additional types of hydrogen in PSMA, mono-partial propyl ester, MW = 1,900, (NR 9), S-A.

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Table 4.7.  $^1\text{H}$ -NMR spectra peaks assignments for PSMA, mono-partial propyl ester, MW = 1, 900 (NR 6).

<b>Functional group</b>	<b>Chemical shift <math>\delta</math> (ppm)</b>	<b>Monomer component</b>	<b>Position (see Figure 4.13)</b>
$\text{CH}_3$ methyl	0.6-1.1ppm	impurities, $\text{CH}_3$	
$\text{CH}_3$ , methyl	1.2-1.8ppm	ester end	e
$\text{CH}_2$ , methylene	2.1-3.0ppm	styrene/mono ester	c, d
CH, methine	3.0-3.4ppm	maleic anhydride	a, b
$\text{O}-\text{CH}_2-\text{CH}_2-\text{CH}_3$	3.4-4.0ppm	partial mono-propyl ester	c-e
C-H aromatics (para, ortho and meta)	6.5-7.6ppm	styrene	a-c
C-H aromatic	8.1ppm	styrene	a-c
solvent peaks	2.04ppm	deuterated acetone.	

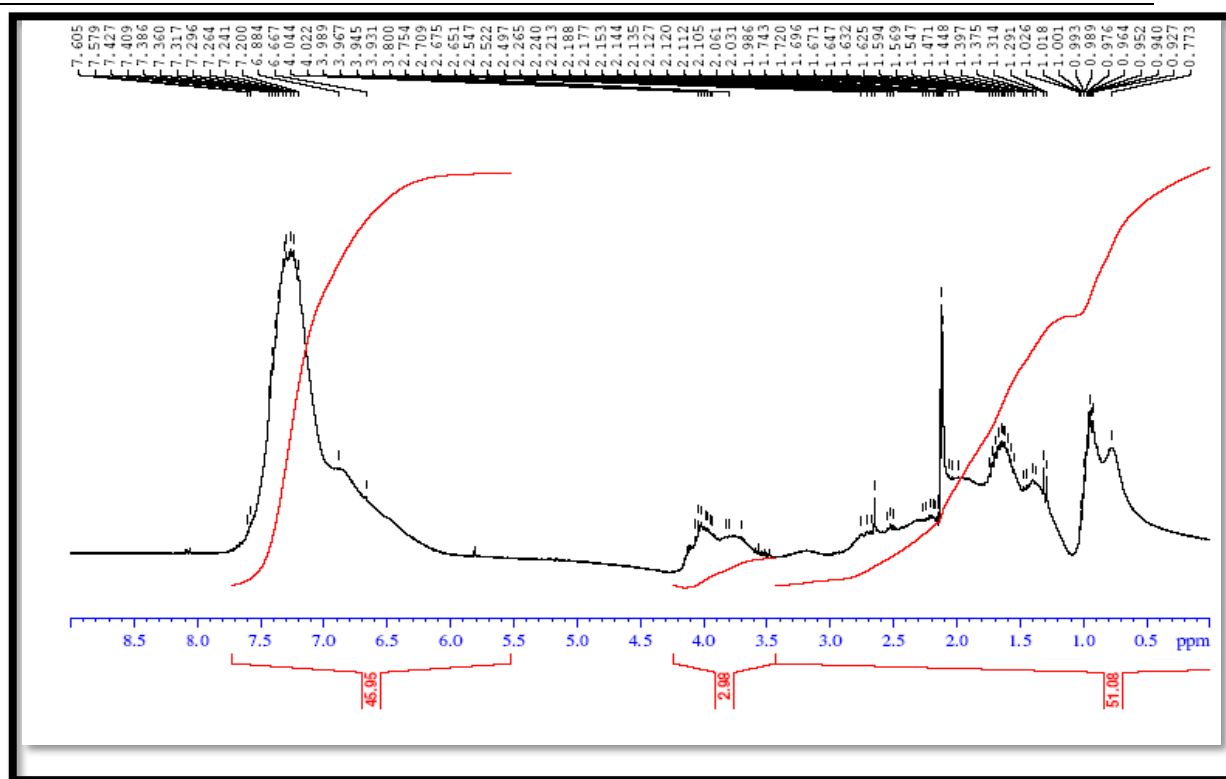


Figure 4.14.  $^1\text{H}$ -NMR spectra of blocky PSMA MW = 1, 900 - mono-partial propyl ester, 2:1 molar ratio, S-A, (NR7) in deuterated acetone ( $\text{d}_6$ ).

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Figure 4.14, shows the presence of the partial propyl ester functionality for the PSMA MW = 1,900 - mono-partial propyl ester, 2:1 molar ratio, S-A, (NR7). Different peaks are present at  $\delta \sim 1.5\text{-}2.1\text{ppm}$  for the methyl end of the ester group and peaks at  $\delta \sim 3.4\text{-}4.1\text{ppm}$ , for the propyl ester functionality.

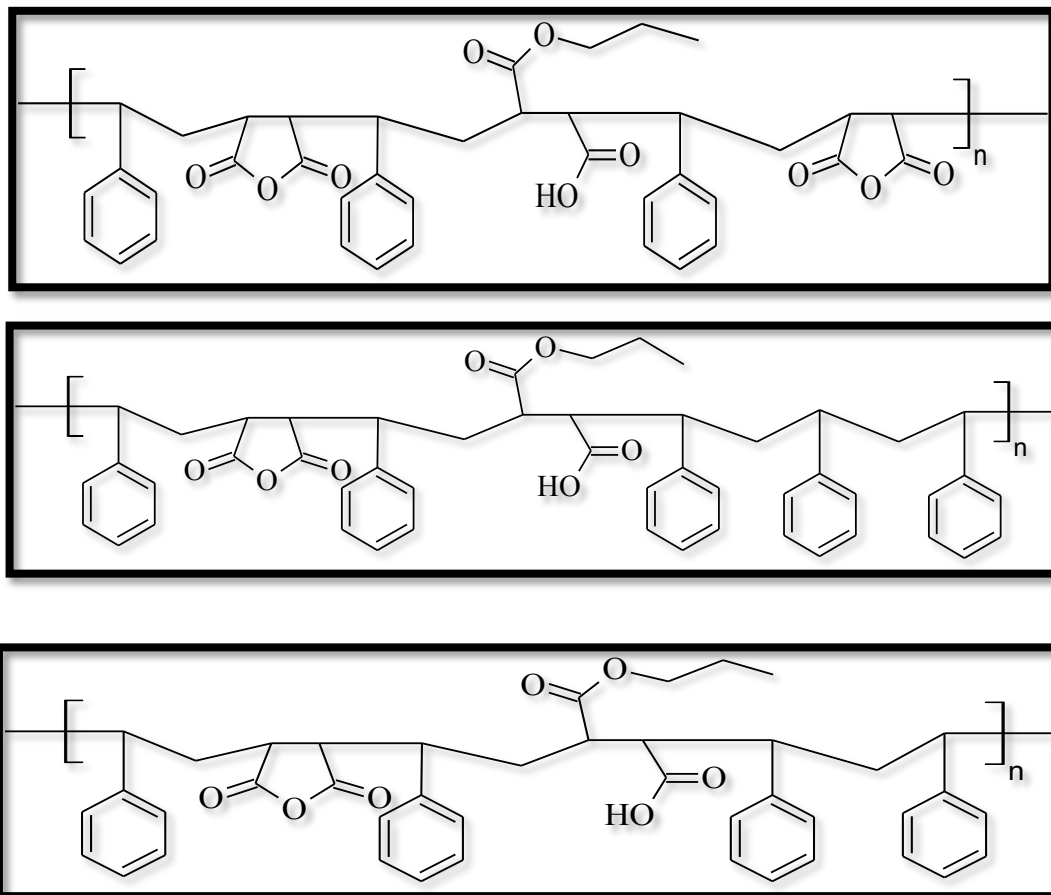


Figure 4.15. Possible monomer sequence distribution for blocky PSMA mono-partial propyl ester based copolymers.

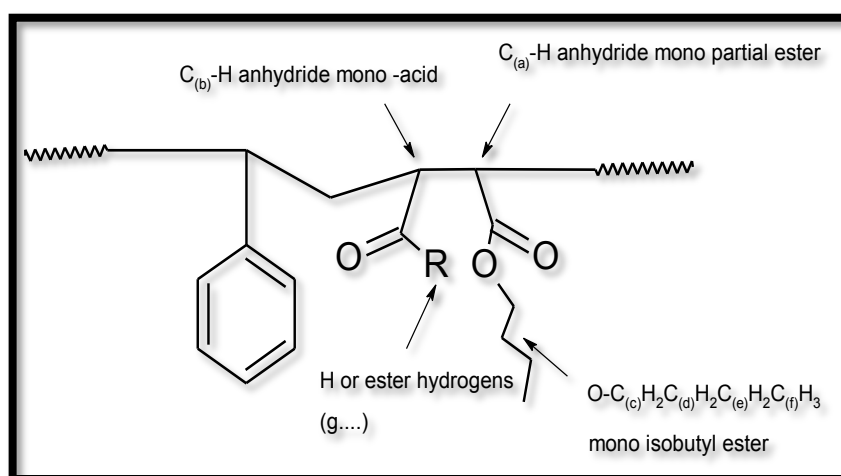



Figure 4.16. Additional types of protons in partial isobutyl/mixed ester PSMA.

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Table 4.8.  $^1\text{H}$ -NMR spectra peaks assignments for PSMA, partial isobutyl/mixed ester.

<b>Functional group</b>	<b>Chemical shift</b>  (ppm)	<b>Monomer component</b>	<b>Position</b> (see Figure 4.16)
$\text{CH}_3$ , methyl	0.5-1.3ppm	$\text{CH}_3$	g, f
$\text{CH}_3$ , methyl	1.5-2.1ppm	$\text{CH}_3$	g, f
$\text{CH}_2$ , methylene	2.1-2.9ppm	$\text{CH}_2$	c
CH, methine	3.0-3.4ppm	maleic anhydride	a-b
$\text{O}-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$ ester	4-4.5ppm	ester/mono partial isobutyl /mixed ester	c-g
C-H (para, ortho and meta)	6.6-7.7ppm	styrene	a-c
C-H aromatic	8ppm	styrene	c
solvent peaks	2.04ppm	deuterated acetone	

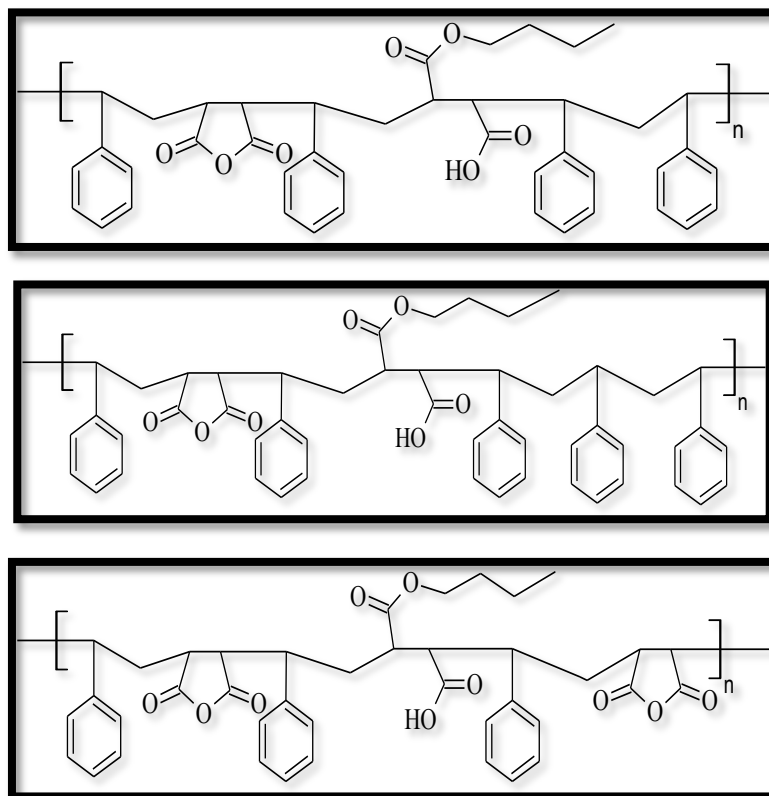


Figure 4.17. Possible monomer sequence distribution for blocky PSMA isobutyl partial ester/mixed, based copolymers.

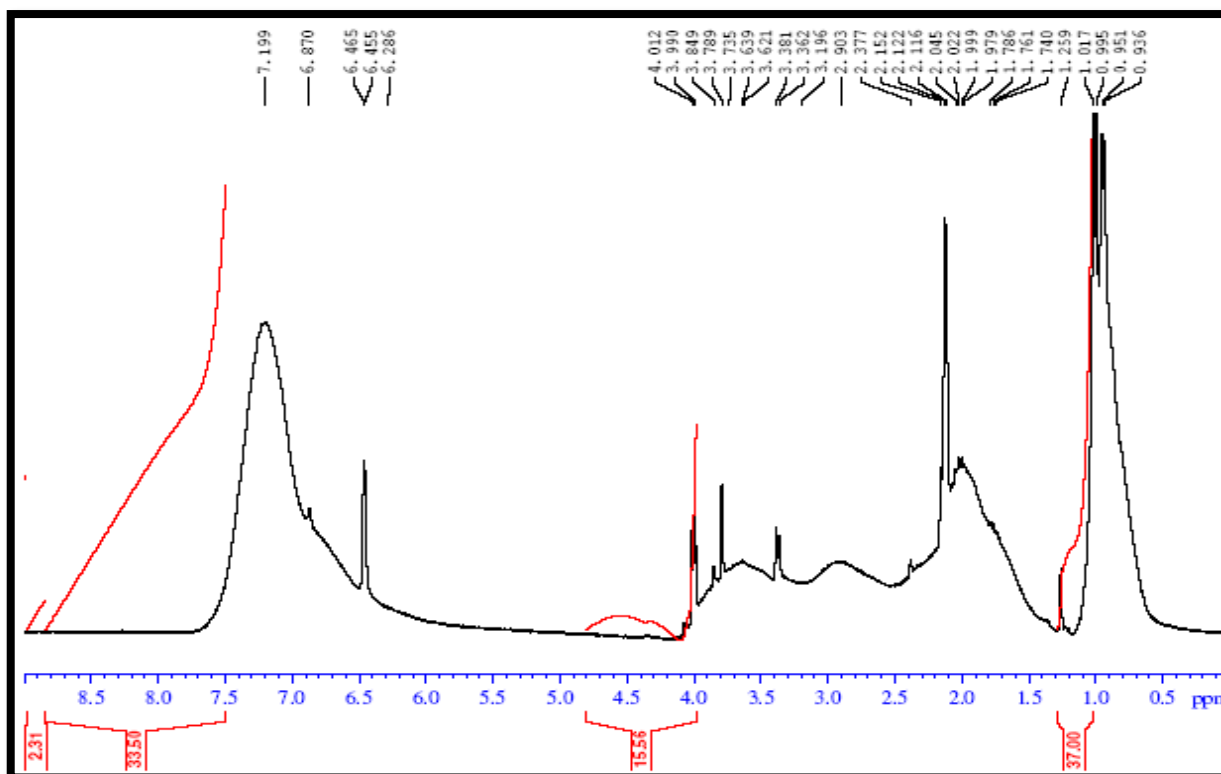


Figure 4.18.  $^1\text{H}$ -NMR spectra of PSMA, MW = 180, 000-partial isobutyl/ mixed ester, 2:1, S-A. (NR9) in deuterated acetone ( $\text{d}_6$ ).

Figure 4.18 above, shows the spectra for the PSMA, MW = 180, 000-partial isobutyl/ mixed ester, 2:1, S-A. (NR9), additional peaks are present at  $\delta \sim 1.5$ -2.1ppm and  $\delta \sim 4$ -4.5ppm, which are due to the partial isobutyl/mixed ester functionality. High-resolution  $^1\text{H}$ -NMR has proved to be a particularly useful tool in the study of the microstructure of polymers in solution, where the extensive molecular motion reduces the effect of long-range interactions and allows the short-range effects to dominate. Although, the NMR spectra for the PSMA-based copolymers are very visually complex due to the overlapping peaks of the styrene and maleic anhydride functional groups, it has been informative in assigning the different  $^1\text{H}$  chemical environments that exist in the various PSMA-based copolymers sought and studied in this thesis. All peaks have been successfully characterised. Line broadening was present as expected for all spectra, due to the viscous polymer solutions under study. Differences in the spectra have been observed; in particular those with higher styrene content (see Figures 4.7- 4.8) and the partial ester PSMA-based copolymers. The fact that many peaks are present with slightly varying chemical shifts, suggests that the PSMA-based systems have a broad microstructure range and many magnetically inequivalent hydrogens are present. Furthermore, it has also been illustrated that the reported copolymer compositions (for the higher styrene content materials) were actually lower in styrene



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content than that reported by manufacturers. Additionally, in these materials more unreacted styrene is present.

These observations thus demonstrate that the reported monomer ratio is based on monomer feed ratio and should not be taken as the actual PSMA-based polymer composition. See Table 4.9 and 4.10 and Figure 4.19 for the evidence obtained. However, in contrast Scott M. Henry *et al* [103] reports that the composition of the hydrolysed PSMA was determined by  $^1\text{H}$  NMR spectroscopy in a deuterated sodium hydroxide solution (1N). All NMR spectroscopy was done on a Bruker DRX499 system. The characteristic aromatic peaks of the styrene subunits (a) 6-7.5 ppm, 5H) and the peaks of the backbone hydrogens from styrene and maleic anhydride (a) 0-3 ppm, 2H from maleic anhydride R-CH-COO-, 3H from styrene R-CH<sub>2</sub>-R, R-CH-Ar were used to determine copolymer composition by solving a set of simultaneous equations. NMR analysis showed the copolymers are composed of 50% styrene and 50% maleic anhydride.

Table 4.9. Estimating styrene-to-maleic anhydride molar ratio by  $^1\text{H}$ -NMR spectra.

<b>PSMA type (see Table 4.1)</b>	<b>Reported % styrene</b>	<b>Calculated by <math>^1\text{H}</math>-NMR (ST-MA)</b>
NR1	50%	46.5%
NR11	66.7%	52.5%
NR12	75%	56%

Table 4.10. The reported styrene content versus calculated via  $^1\text{H}$ -NMR analysis.

<b>Monomer feed ratio (styrene-to- maleic anhydride)</b>	<b>%Styrene by <math>^1\text{H}</math>-NMR</b>	<b>% Maleic anhydride by <math>^1\text{H}</math>-NMR</b>	<b>Polymer composition by <math>^1\text{H}</math>-NMR (styrene-to- maleic anhydride ratio)</b>
1:1	46.47%	53.35%	1:1.14
2:1	52.46%	47.54%	1.10:1.0
3:1	55.69%	44.31%	1.26:1.0
1:1 - partial methyl ester (15%)	48.61%	51.39%	1:1.06
2:2 - partial propyl ester (50%)	45.95%	54.06%	1:1.18

The explanation for the differences observed in the monomer feed ratio estimated by  $^1\text{H}$ -NMR and that reported by the manufacturer could be due to the method utilised to calculate the molar ratio or may simply be due to the fact the molar ratio reported is

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simply the molar ratio utilised to synthesis the copolymers as opposed to the actual polymer composition.

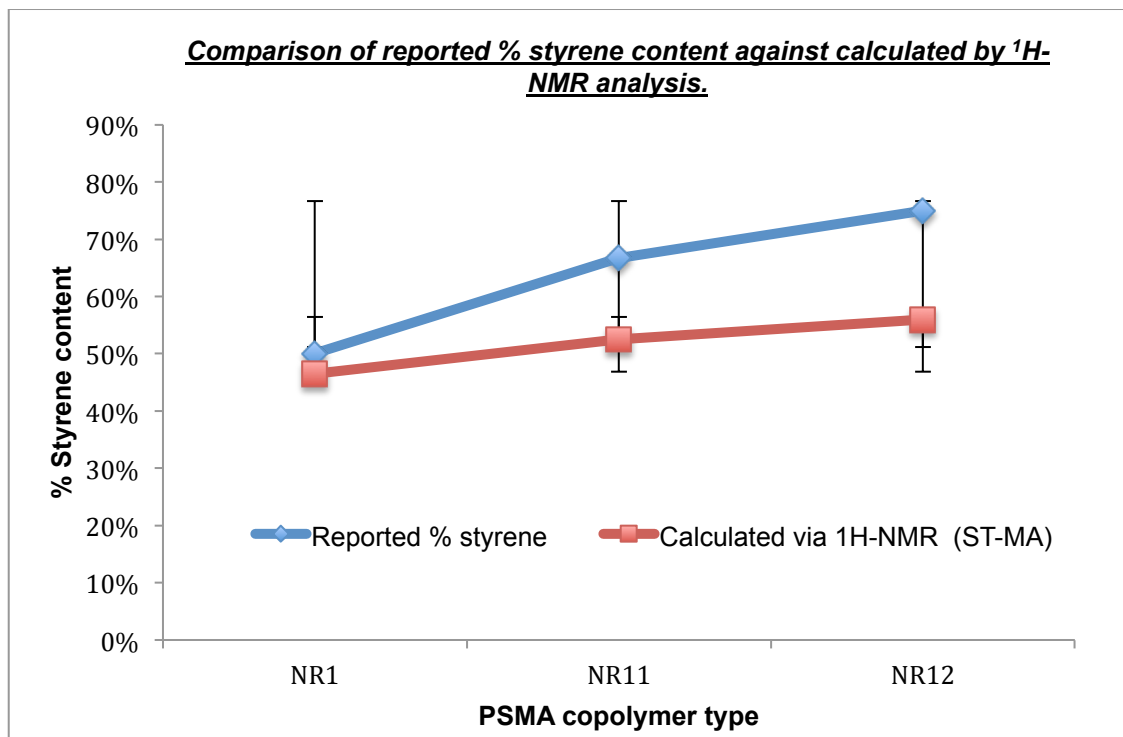


Figure 4.19. Comparison of reported % styrene content against calculated by  $^1\text{H}$ -NMR analysis.

The rates of relaxation of nuclei are important, the spin-lattice relaxation, otherwise known as longitudinal relaxation, determines the rate at which net adsorption can occur. The mean half-life of the spin-lattice relaxation process is designated  $T_1$ , and that of the spin-spin relaxation, otherwise known as transverse relaxation process  $T_2$ . If  $T_1$  and  $T_2$  are small, then the lifetime of an excited nucleus is short, and it has been found that this gives rise to very broad adsorption lines in the NMR spectrum. If  $T_1$  and  $T_2$  are large, perhaps of the order of 1 second, then sharp spectral lines arise. For nonviscous liquids (and that includes solutions of solid in non-viscous solvents) molecular orientations are random, and transfer of energy by spin-lattice relaxation is inefficient. In consequence,  $T_1$  is large, and this is one of the reasons why sharp signals are obtained in NMR studies on non-viscous systems. The approximate value of the copolymer composition can be calculated from  $^1\text{H}$ -NMR integral peaks (see Table 4.9). However, a more accurate quantitative analysis can be obtained by measuring the  $T_1$  values for the polymer under study, (so that the correct time delay can be set) when collecting the FID (free induction decay). These phenomena need to be taken into consideration whilst interpreting the data compiled in Table 4.9 and 4.10. Nonetheless,  $^1\text{H}$ -NMR spectra analysis revealed the presence of additional mono-

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partial ester functionalities. See Figures 4.10, 4.12 and 4.14, for the NMR spectra representing the esterified type PSMA-based polymers studied in this chapter. The  $^1\text{H}$ -NMR spectra of styrene-maleic anhydride copolymers are visually complicated due to overlapping peaks and the complex NMR phenomena of polymers. However, despite these limitations just simple  $^1\text{H}$ -NMR has been successful in revealing the structural differences and most importantly illustrating that even the 1:1 molar ratio PSMA-based copolymers, MW 1,600 (NR1) are not perfectly alternating in contrast to what has been reported in the literature.

### 4.2.2 Edited $^{13}\text{C}$ -NMR (PENDANT) NMR analysis of PSMA-based copolymers

$^{13}\text{C}$ -NMR (PENDANT) NMR analysis was undertaken to investigate the  $^{13}\text{C}$  chemical environments in PSMA-based copolymers sought. See Figure 4.20, below for all the types of carbons in a simple PSMA repeat unit.

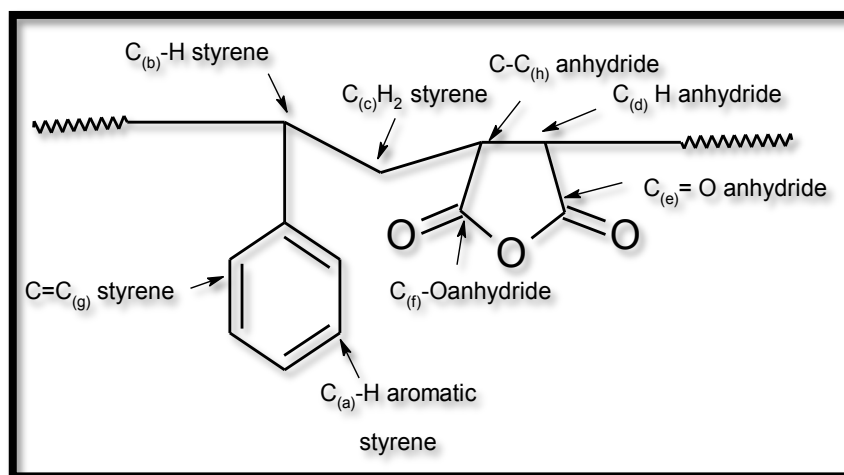


Figure 4.20. Types of carbon in a simple PSMA repeat unit (full anhydride)

From first glance it may appear that assignment e and f have the same chemical environment, however this is only the case for an alternating triad. This basic differentiation can distinguish that PSMA-based copolymers are not perfectly alternating, as already reported from the  $^1\text{H}$ -NMR analysis.  $^{13}\text{C}$ -NMR spectroscopy is a very useful method to assess the different sequential distributions of adjacent configurational units that are called dyads, triads, tetrads and pentads etc. Edited  $^{13}\text{C}$  NMR PENDANT (pulse sequence Polarization Enhancement Nurtured during Attached Nucleus Testing) [78-80] was utilised to assign the types of carbons in the PSMA-based copolymers sought. This technique circumvents the problem of 'lost quaternary carbons' while giving maximum signal intensity by polarisation transfer. It was used to

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enable the C-H couplings to be resolved into methine and methyl, methylene and quaternary groups. The detection was phased so that methylene and quaternary carbons generate a negative signal while methine and methyl groups generate a positive signal. The peaks associated with the solvent utilised (deuterated acetone in this case) was phased negatively [78-80].

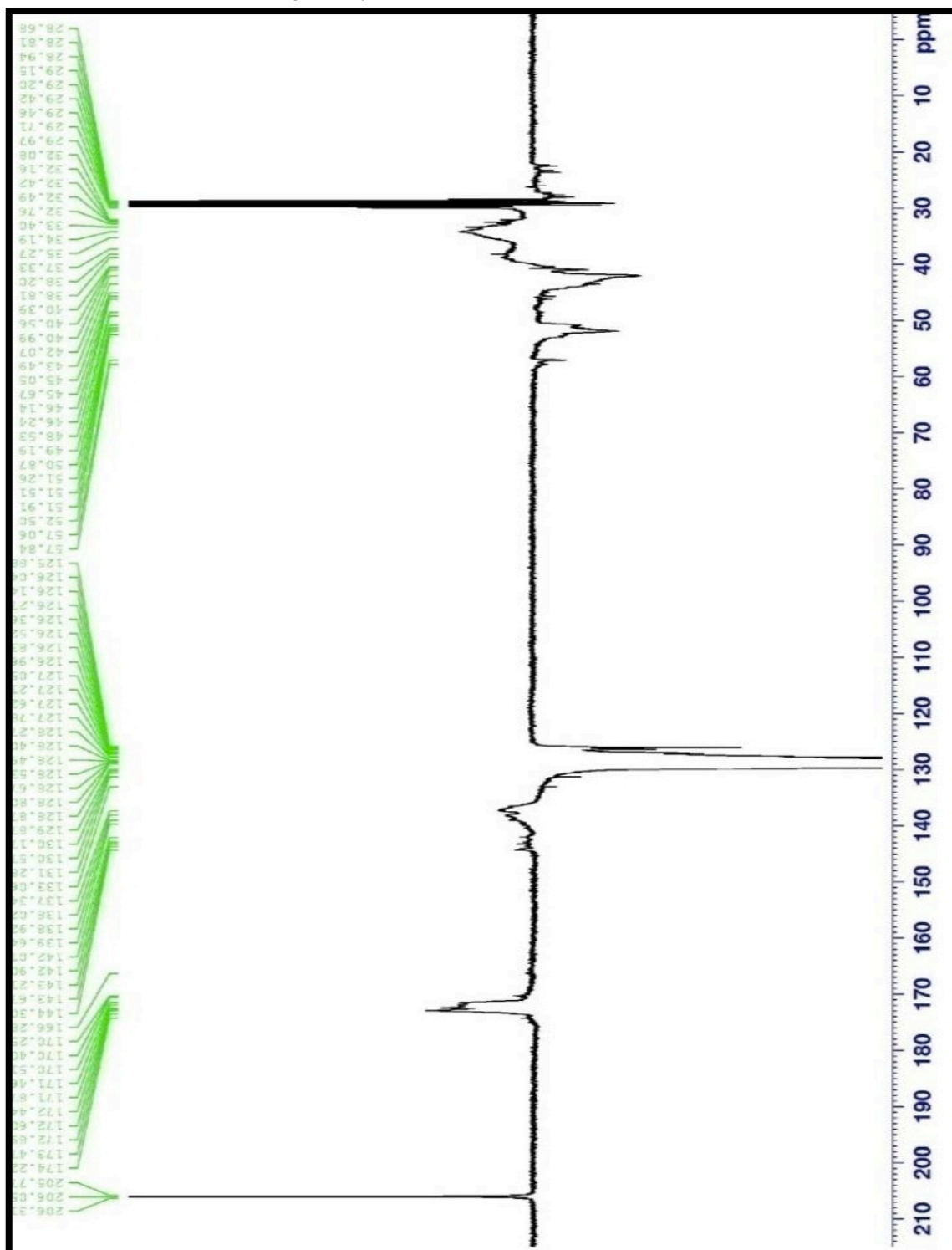


Figure 4.21. Edited  $^{13}\text{C}$  (PENDANT) spectra of POL - PSMA MW = 1, 600 (1:1), NR1, in deuterated acetone ( $\text{d}_6$ ).

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Figure 4.21 above depicts the different types of carbon chemical environments for PSMA-based. Table 4.11 below summarizes and identifies these accordingly, in accordance to the assignments reported in NMR analytical handbooks relating to the chemical shifts in relation to the respected functional group and chemical environment associated with the respected type of carbon.

Table 4.11. A summary of  $^{13}\text{C}$ -peaks for PSMA-POL - MW 1,600 (NR1).

<b>Functional group</b>	<b>Chemical shift <math>\delta</math> ppm (phase +ve/-ve)</b>	<b>Monomer component/position on Figure 4.20</b>
deuterated acetone	29ppm (-ve)	solvent
<b><math>\text{CH}_2</math>, methylene - C1</b>	<b>32-39ppm (-ve)</b>	<b>alkene styrene (c)</b>
CH, methine	38-44ppm (+ve)	maleic anhydride (d)
C-O, anhydride ring	50-56ppm (-ve)	maleic anhydride (f)
C=C, aromatic	126-135ppm (+ve)	styrene (a-b)
C=O, carboxylic	170-174ppm (-ve)	maleic anhydride (f)
<b>quaternary styrene - C7</b>	<b>134-145ppm (+ve)</b>	<b>styrene (b)</b>
deuterated acetone	206ppm (-ve)	solvent

Table 4.11 above depicts that overall many magnetically inequivalent carbons are present in the PSMA 1:1 (molar ratio) MW 1,600 - NR1. This strongly suggests and is in agreement with the  $^1\text{H}$ -NMR data generated, that a broad microstructure range is present in all PSMA-based copolymer systems. Specifically, for the PSMA-POL, MW 1,600 (NR1) copolymer, the methylene carbons (see position 'c' in Figure 4.20) appear at  $\delta \sim 32\text{-}39\text{ppm}$ , the methine carbons (see position 'd' on Figure 4.20) appear at  $\delta \sim 38\text{-}44\text{ppm}$ . The C-O anhydride ring functionality (see position 'f' on Figure 4.20) is ascribed at  $\delta \sim 38\text{-}44\text{ppm}$ . The carboxylic maleic anhydride (C=O) peaks (see position 'f' on Figure 4.20) shows at  $\delta \sim 170\text{-}174\text{ppm}$ , and the quaternary carbons have been assigned at  $\delta \sim 134\text{-}145\text{ppm}$ . The signal for C atoms from C=O groups of MA units appears for different copolymers, at  $\delta \sim 171\text{-}173\text{ppm}$ ; 172-174; 173.5-171.8 and 173-178ppm, depending on the anhydride ring environment.

Figure 4.22, depicts the Edited  $^{13}\text{C}$  (PENDANT) spectra of PSMA MW = 350, 000 (1:1), partial mono-methyl ester. New peaks have been identified for the partial methyl ester functionality.

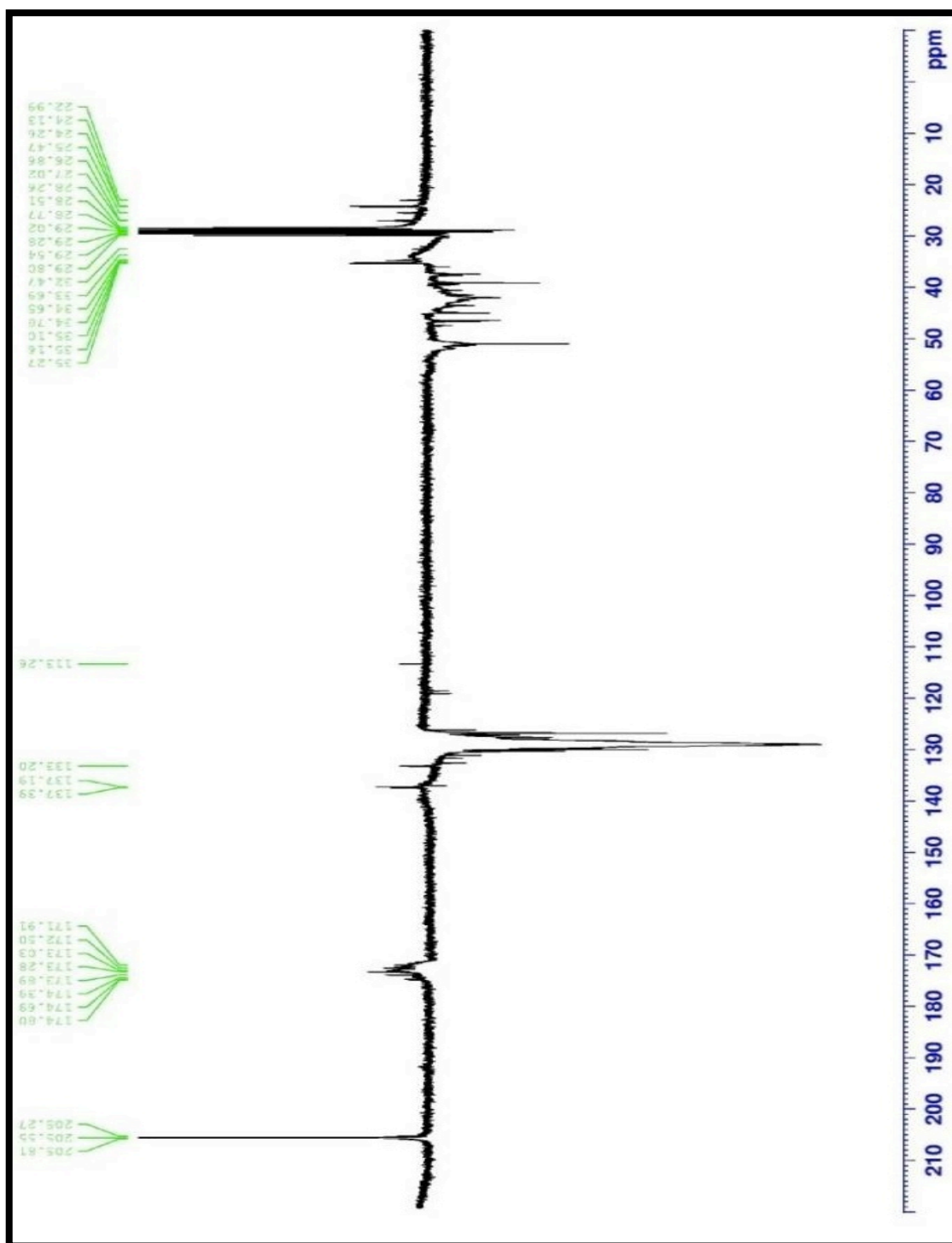


Figure 4.22. Edited  $^{13}\text{C}$  (PENDANT) spectra of PSMA MW = 350, 000 (1:1), partial mono-methyl ester, showing the presence of methyl ester functionality, in deuterated acetone ( $\text{d}_6$ ), NR6, S-A.

Figure 4.23, illustrates the different type of carbons that are additionally present in PSMA MW = 350, 000 (1:1), partial mono-methyl ester copolymer.

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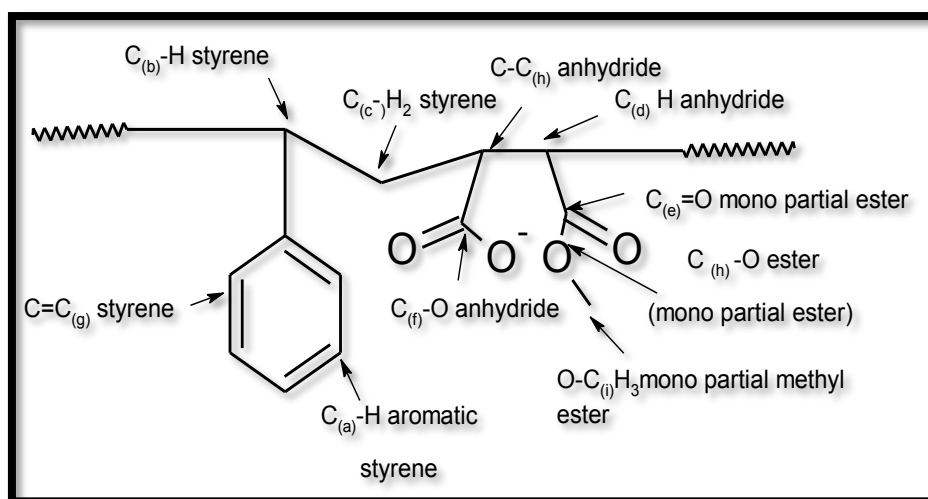


Figure 4.23. Types of carbon chemical environments in a mono-partial methyl ester PSMA-based copolymer.

Table 4.12. A summary of  $^{13}\text{C}$ -peaks for PSMA, MW = 350, 000 (1:1), partial mono-methyl ester, NR6, S-A.

<b>Functional group</b>	<b>Chemical shift <math>\delta</math> ppm (phase)</b>	<b>Monomer component/functional group</b>
deuterated acetone	29ppm (-ve)	solvent
<u>C-C, <math>\text{CH}_3</math></u>	<u>25-30ppm</u>	<u>partial methyl ester</u>
<u>methyl ester (<math>\text{O}-\text{CH}_3</math>)</u>	<u>40-60 ppm</u>	<u>partial methyl ester</u>
<b><math>\text{CH}_2</math>, methylene - C1</b>	<b>32-43ppm (-ve)</b>	<b>alkene styrene</b>
CH, methine	38-44ppm (+ve)	maleic anhydride
C-O, anhydride ring	50-56ppm (-ve)	maleic anhydride
<u>methyl ester</u>	<u>112ppm</u>	<u>partial methyl ester</u>
C=C, aromatic	126-135ppm (+ve)	styrene
C=O, carboxylic	170-174ppm (-ve)	maleic anhydride
<b>quaternary styrene - C7</b>	<b>134-145ppm (+ve)</b>	<b>styrene</b>
deuterated acetone	206ppm (-ve)	solvent

In Table 4.12 above, the underlined rows have been identified as the partial methyl ester peaks. Peaks at  $\delta \sim 25\text{-}30\text{ppm}$ ,  $\delta \sim 40\text{-}60\text{ ppm}$ ,  $\delta \sim 40\text{-}60\text{ppm}$  and at  $\delta \sim 112\text{ppm}$  represent the partial methyl ester functionality. This provides strong evidence for the presence of partial methyl ester functionality in PSMA-based NR6. The other peaks that vary are; the methylene peaks, these are slightly shifted and appears at 32-43ppm, as opposed to 32-39ppm in the spectra for NR1. The quaternary styrene carbons are

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slightly shifted too and also vary. The peaks appear at  $\delta \sim 134$ -145ppm, as opposed to  $\delta \sim 134$ -145ppm, in PSMA-based copolymer NR1.

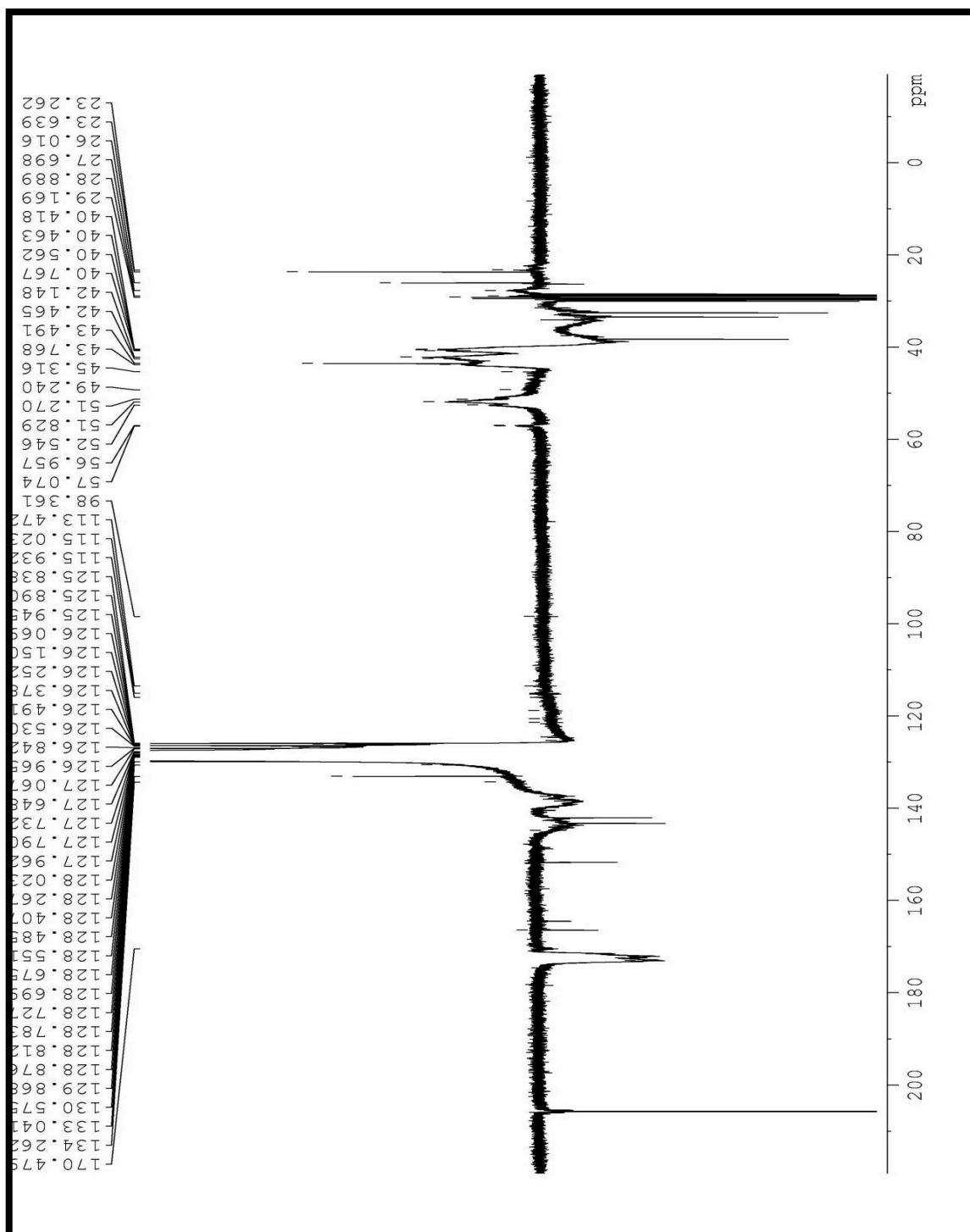


Figure 4.24. Edited  $^{13}\text{C}$  (PENDANT) spectra of PSMA (3:1) MW 9, 500, in deuterated acetone ( $\text{d}_6$ ), NR 3, POL.



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Figure 4.24, depicts the carbon chemical environments for PSMA, MW 9,500, 3:1 molar ratio, POL. As you can see, differences are observed, as expected. Table 4.13, summarises the differences in the carbon chemical environments observed.

Table 4.13. A summary of  $^{13}\text{C}$ -peaks PSMA (3:1) MW 9, 500, in deuterated acetone ( $\text{d}_6$ ), NR 3, POL.

<b><i>Functional group</i></b>	<b><i>Chemical shift <math>\delta</math> ppm (phase +ve/-ve)</i></b>	<b><i>Monomer component</i></b>
deuterated acetone	29ppm (-ve)	solvent
impurities	20-30ppm	not part of molecule
<b><i>CH<sub>2</sub>, methylene - C1</i></b>	<b><i>32-41ppm (-ve)</i></b>	<b><i>alkene styrene</i></b>
CH, methine	40-44ppm (+ve)	maleic anhydride
C-O, anhydride ring	50-54ppm (-ve)	maleic anhydride
C=C, aromatic	126-135ppm (+ve)	styrene
C=O, carboxylic	170-177ppm	maleic anhydride
<b><i>quaternary styrene - C7</i></b>	<b><i>138-150ppm (+ve)</i></b>	<b><i>styrene</i></b>
deuterated acetone	206ppm (-ve)	solvent

Table 4.13 above, summaries all peaks representing the different carbon chemical environments for the NR3 PSMA-based copolymer. The unique features are shown as bold and italic and illustrate that the methylene and quaternary carbon chemical environment are slightly different. The quaternary styrene peaks are more so shifted. This is expected, as the styrene content for this PMAS-based copolymer is the greatest.

Polymers are frequently synthesised from asymmetric monomers giving rise to the possibility that the incoming monomer can add to the chain in head-to-head or tail-to-tail orientation. A head-to-head junction with no accompanying tail-to-tail unit will also rise from the recombination of growing chain radicals, but in this case there can be only one such per unit per chain. They have proposed the terms isoregic and syndioregic to describe head-to-tail and head-to-head-tail-to-tail sequences, respectively [73-85]. For the purposes of NMR measurements three consecutive monomer units in a chain are considered to define a configuration and called a triad. One of the most significant applications of NMR to macromolecules is the observation and quantitative measurement of chain microstructure. This term embraces those features of polymer chains which are fixed by their covalent structure, and is generally understood to

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include the following; head-to-tail versus head-to-head-tail-to-tail isomerism, i.e., regioisomerism; stereochemical configuration; geometrical isomerism; and branching and crosslinking. Peaks from different microstructures can be resolved in the NMR spectrum, providing a detailed and quantitative characterisation of chain microstructure.

Although literature reports that PSMA-based copolymer synthesis via equal equimolar quantities of styrene and maleic anhydride should generate alternating copolymers, NMR analysis studied in this thesis reveals, (in particular edited  $^{13}\text{C}$ -NMR spectroscopy acquired), that even the reported 1:1 molar ratio PSMA-based copolymers, are in fact random and thus atactic, in terms of polymer microstructure.

When a radical attacks an asymmetric vinyl monomer two modes of addition are possible. The actual mode of addition depends on stability of product and the possible steric hinderance of the approach of the radical caused by a large X group (aromatic in this case). Vinyl polymers ( $-\text{CH}_2-\text{CHX}-$ ) may show different configurations with respect to the head (CHX) and tail ( $\text{CH}_2$ : head-to-head, with  $-\text{CHX}$  bonded to  $-\text{CH}_2$  and head-to-head-tail-to-tail, with  $-\text{CHX}$  bonded to  $-\text{CHX}$  followed by  $-\text{CH}_2$  bonded to  $-\text{CH}_2$  [93]. See Figure 4.25 for all possible structures. The mode of addition depends on factors such as steric hinderance, on approach to the macro-radical, caused by a large group (X) in the molecule. Head-to-tail is highly favoured, possibly due to resonance stabilisation, although tail-to-tail or head-to-head may occur when termination by combination predominates.

In conclusion,  $^{13}\text{C}$  NMR studies detects region-irregular structures, atactic, random monomer sequence distribution, giving rise to statistical PSMA-based copolymers, as apposed to regioregular structures, which would be expected for perfectly alternating PSMA-based systems.

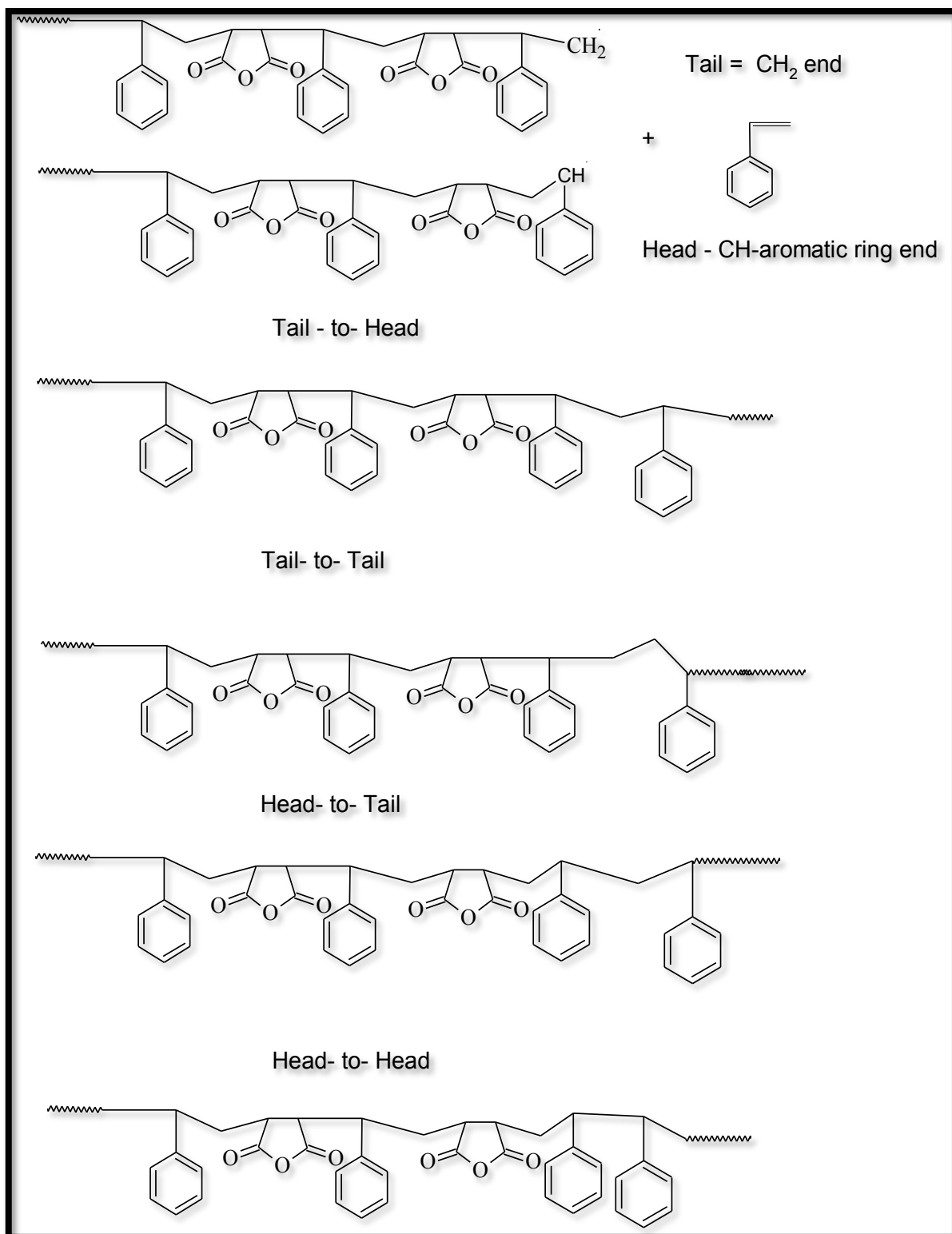


Figure 4.25. Head-to-Tail versus head-to-head-tail-to-tail-isomerism-regio-isomerism in PSMA-based copolymers (the difference in head to tail and tail-to-head being the orientation of the incoming monomer).

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Edited  $^{13}\text{C}$  NMR (PENDANT) acquisition of the PSMA MW = 1,600 provides strong evidence for the presence of regioisomerism in PSMA systems, as there is more than one peak for the methylene,  $\text{CH}_2$  peaks, due to the magnetic inequivalent  $\text{CH}_2$  carbons present in the polymer chains. See Figure 4.25 for all possible variations in isomerism orientation and the subtle differences that occur along the PSMA chain.

$^{13}\text{C}$  NMR spectroscopy has proved to be a very useful technique for the structural analysis of the various PSMA-based copolymers, because of the simplicity of spectra and the wide range of chemical shifts. This method has identified non-alternating distribution of monomers for all PSMA-based systems, previously reported as perfectly alternating. This must be expected for PSMA-based copolymers synthesised from non-equimolar monomer feeds/or at higher reaction temperatures, which possess a far from 1:1 molar ratio composition. For PSMA-based copolymers of varying mole fractions, the copolymerisation conditions are also incompletely specified.

N.T.Hieu Ha *et al* [105] reports that the aromatic 'next to polymer chain' carbon (C7) of donor monomer (1) units in PSMA-based copolymers is sensitive to the unit 1-centred triad sequence distribution. The acceptor monomer is referred to as 0. The chemical shifts of the non-alternating (111), semi-alternating (011 and 110) and alternating (010) triad sub-peaks were assigned in ppm. In the homopolymer of styrene, the carbon which has the special 'status' of a quaternary carbon and the special 'position' as a 'next to polymer chain' aromatic carbon in poly(styrene) was reported to split into three distinct groups, which were assigned to isotactic(mm), heterotactic(mr) and syndiotactic(rr). For the styrene-containing copolymers, the effect of copolymer sequences besides that of stereochemical configurations are considered. Maleic anhydride (MA or 0) is a strong electron acceptor and does not homopolymerise under ordinary conditions but copolymerises with styrene (ST) to form the alternating MA (0)-ST (1) copolymers. This evidence serves us in the assignments of NMR peaks; the (010) peak is much more intense than the other two peaks. The quaternary aromatic 'next to polymer chain' carbon (C7) and the methylene ( $-\text{CH}_2-$ ) 'polymer chain' carbon (C1) of ST (1) units were reported to be sensitive to the ST (1) - centered triad sequence distribution observed by the  $^{13}\text{C}$  NMR spectra. The authors present the  $^{13}\text{C}$  NMR spectrum of an alternating MA-ST copolymer, because MA and ST alternate strongly, the non-alternating (111 or ST-ST-ST) sequences are shown only when the MA mole fraction ( $F_0$  in feed is very small ( $F_0$  0.01-0.05). When  $F_0 = 0.90$ , the fully alternating copolymer was formed, i.e. only the resonance of the alternating (010) triad sub-peak appears in the spectra of C7 and C1. The resonance of C7 carbon appears

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in a shorter range [147.5-136.5 .11(ppm)] than the range for C1 carbon [47-33(ppm)]. In the spectra of C7 carbon, the non alternating (111) and semi-alternating (011 and 110) triad sub-peaks overlap by around 1ppm at the most when the MA mole fraction in the feed is very small ( $F_0 = 0.01 - 0.02$ ); while they quite clearly separated in the C1 spectra. The qualitative determination or the chemical shift assignments for the three ST (1) centered triad sub-peaks in the spectra of C7 carbon is confirmed by the consistence of the appearances of the three triad sub-peaks from both spectra of C1 and C7 [105].

Table 4.14. The chemical shifts of the non-alternating (111), semi-alternating (011 and 110) and alternating (010) triad sub-peaks for methylene  $\text{CH}_2/\text{C1}$  assigned in ppm for PSMA-based copolymers[105].

<b><i>Triad sequence sub-peaks for quaternary St/C7</i></b>	<b><i>Chemical shift (PPM)</i></b>
alternating - 010 (ST-MA-ST)	136.5ppm-141.5ppm
semi-alternating - 011 or 110 (ST-ST-MA) or MA-ST-ST	141.5-145.5ppm
non-alternating (ST-ST-ST)	148 ppm

Table 4.15. The chemical shifts of the non-alternating (111), semi-alternating (011 and 110) and alternating (010) triad sub-peaks for quaternary styrene/C1 carbon assigned in ppm in PSMA-based copolymers[105].

<b><i>Triad sequence sub-peaks for <math>\text{CH}_2/\text{C1}</math></i></b>	<b><i>Chemical shift (PPM)</i></b>
alternating - 010 (ST-MA-ST)	32-37ppm
semi-alternating - 011 or 110 (ST-ST-MA) or MA-ST-ST	37-42ppm
non-alternating - 111 (ST-ST-ST)	42-47ppm

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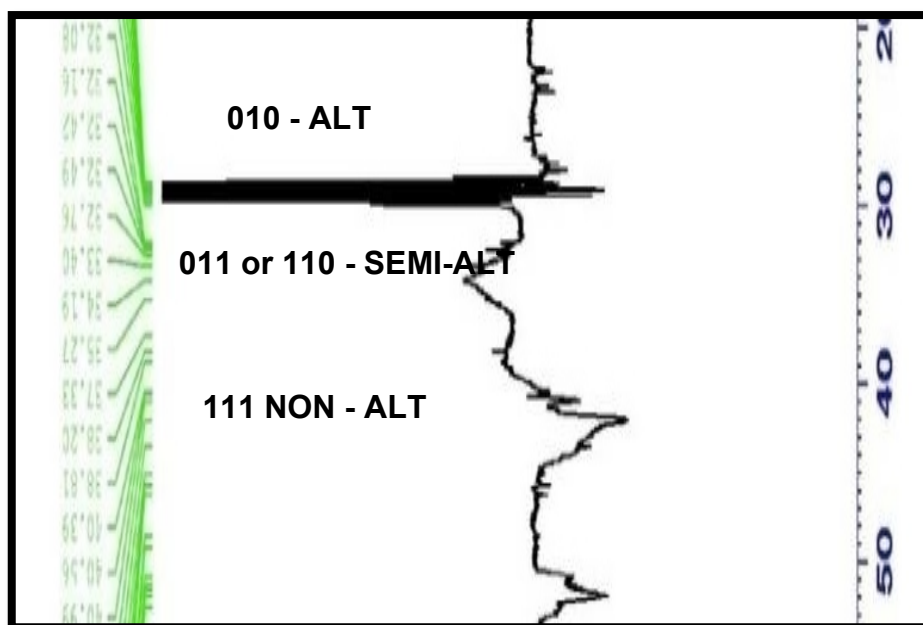
The monomer feed ratio/polymer composition determines the distribution of the monomer sequence, as illustrated in Figures 4.26, 4.27 and 4.28. All three spectra attained depict that, alternating, semi-alternating and non-alternating triad sequences are present in all three PSMA-based copolymers systems.

By examining and relatively comparing the  $-CH_2-$  methylene chemical shift region for PSMA 1:1 molar ratio, MW 1,600 (NR1) with PSMA 3:1 molar ratio MW 9,500 (NR3) Figure 4.26, highlights that the triad sequence monomer distribution content varies between the two PSMA-based copolymers. The  $^{13}C$ -NMR spectra for the higher styrene content copolymer (NR3), has more prominent peaks that are representative of the semi-alternating (37-42ppm) and non-alternating triad (42-47ppm) monomer sequence distribution. Nonetheless, the alternating triad peak is also present (32-37ppm) - see Figure 4.26. In comparison, the  $^{13}C$ -NMR spectra for the 1:1 molar ratio PSMA, MW 1,600 - NR1 has peaks for all semi-alternating, non-alternating and alternating triad monomer sequences which seem all fairly well represented, this agrees well with the general thesis finding regarding the microstructure of PSMA-based copolymers not being perfectly alternating, in contrary to that reported previously by many other researchers.

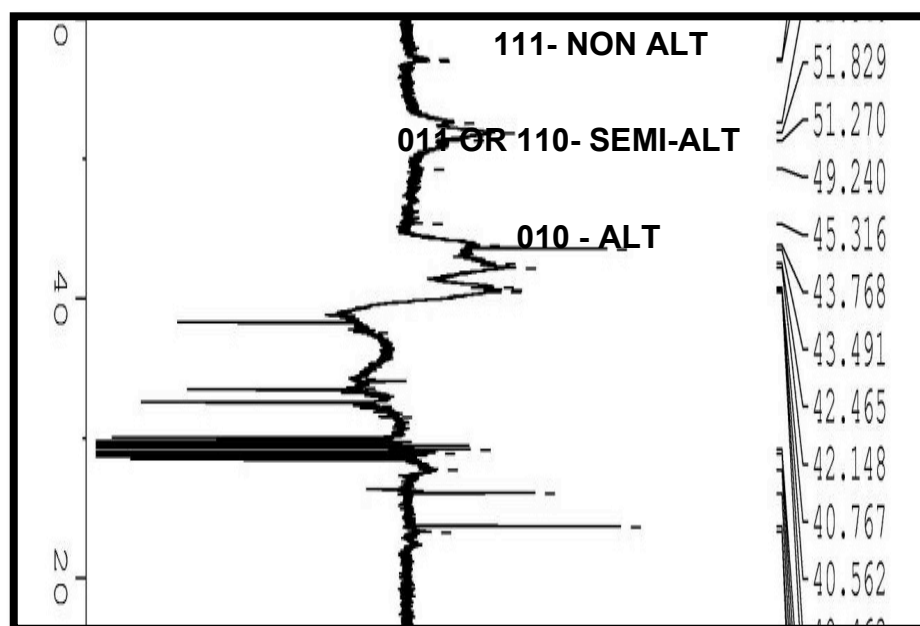
Strong evidence is also presented in Figure 4.26 it shows that the higher styrene content copolymers have a greater concentration of blocky ST-ST (non-alternating) monomer sequence distribution. Figure 4.27 shows comparison of the quaternary C7 chemical environment, the peaks are very intense and look similar in nature but are very busy. It is difficult to clearly distinguish the three types of triad peaks.

The triad sensitive regions for the  $^{13}C$ -NMR spectra of the partial methyl ester, 10-15%, MW 350,000 PSMA-based copolymer (NR6) can be visualised in Figures 4.28. All three triad monomer sequences are present for the quaternary and methylene carbon chemical shift regions.

Furthermore, it would be desirable to analyse by the  $^{13}C$ -NMR DEPT methods some copolymers with 1:1 molar ratios but different monomer distributions. At the same time, lower contents of alternating triads can indicate a mechanism of copolymerisation involving participation of both charge transfer complexes and free monomers in the propagation process.



**A – NR1**

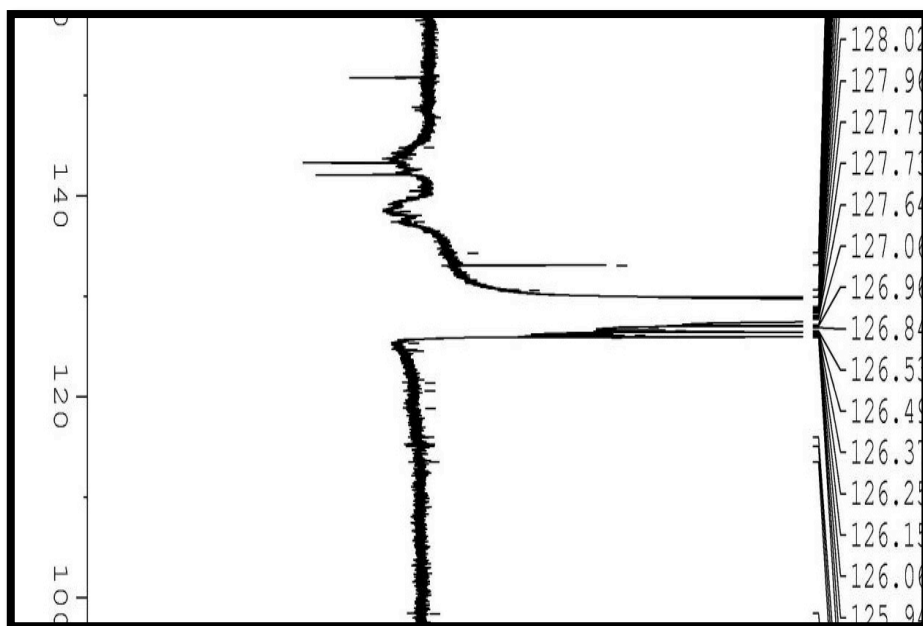


**B – NR3**

Figure 4.26. Methylene CH<sub>2</sub> region for 1:1 (A) and 3:1(B) monomer feed ratio PSMA-based copolymers.

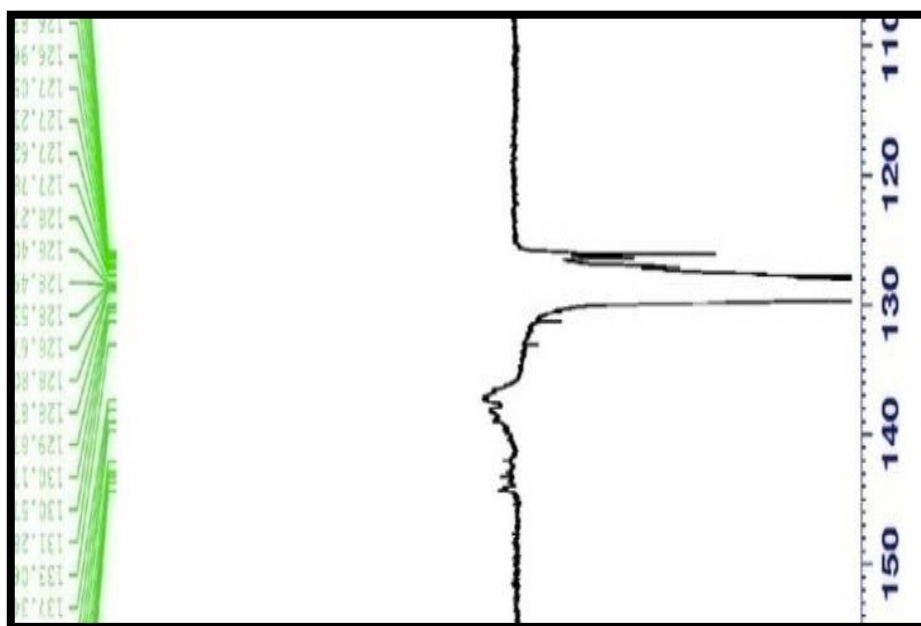
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**010 - ALT**  
**010 - SEMI ALT**  
**111 - NON ALT**



**A-NR3**

**010 - ALT**  
**010 - SEMI ALT**  
**111 - NON ALT**



**B-NR1**

Figure 4.27. Quaternary C7 for 3:1 (A) and 1:1(B) monomer feed ratio PSMA-based copolymers.

Please note that assignments vary due to spectra processed under slightly different conditions.



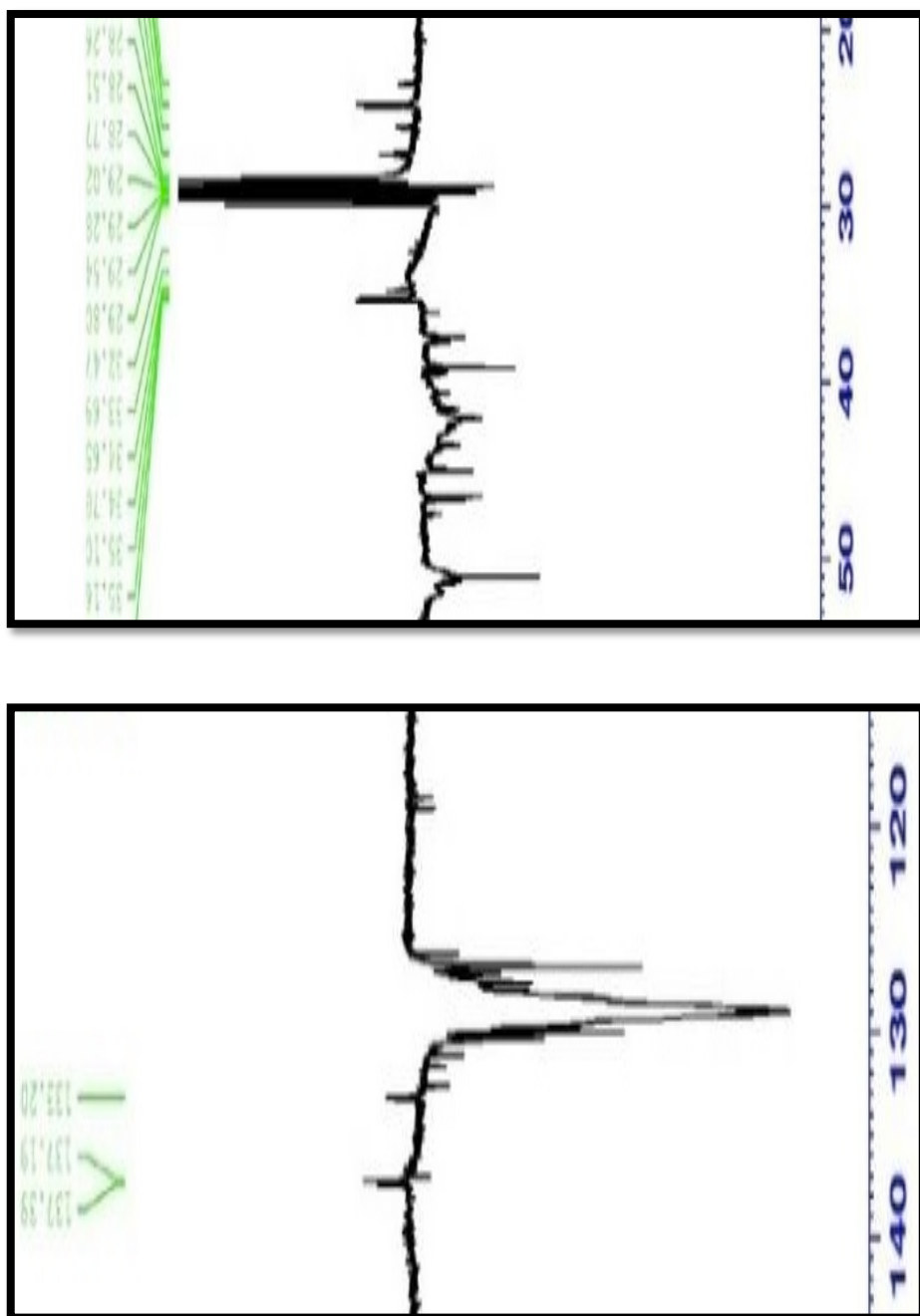


Figure 4.28. Quaternary styrene C7/and methylene  $\text{CH}_2$  region for  $^{13}\text{C}$ -NMR spectra for PSMA 350, 000 -10-15% partial methyl ester - NR6.

#### 4.2.3 2D C-H correlation (HSQC) analysis

Hetero Nuclear Single Quantum Coherence (HSQC) spectroscopy provides the short-range  $^1\text{J}_{\text{CH}}$  connectivities and thereby applies only to those C atoms, which are linked to H and not to non-protonated C atoms. The experiment uses the INEPT sequence to generate transverse X magnetization which evolves and is then transferred back to the proton by an INEPT step in reverse [83]. HSQC (a carbon hydrogen correlation

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experiment) was carried out to clarify the assignments made for the  $^1\text{H}$  and  $^{13}\text{C}$ -NMR spectra collected. This was necessary in order to define the precise locations of the protons, as the hydrogen and carbon NMR acquired was of poor resolution, due to the complexity of overlapping peaks and complex NMR phenomena for spectra of viscous polymer solutions. This technique was used to assign which carbons are chemical bonded to which hydrogens. Tables 4.16 and 4.17 provides strong evidence that the peaks are not due to any impurities or solvents.

Table 4.16. HSQC peak assignments for PSMA (POL, 1:1 MW 1,600) NR1.

<b><math>^1\text{H}</math> chemical shift /ppm</b>	<b><math>^{13}\text{C}</math> chemical shift/ppm</b>	<b>Correlated aliphatic assignments</b>
2.1-2.7ppm	30-35ppm	$\text{CH}_2$ - styrene
3.1-3.7ppm	38-43 ppm	CH - maleic anhydride
6.2-7.7ppm	120-130ppm	$\text{C}_6\text{H}_5$ aromatic styrene

Table 4.16, above summarises the C-H correlations observed for the PSMA-based copolymer NR1, depicted in Figure 4.29. As you can see below in Table 4.17, the C-H correlation assigned peaks for PSMA-based copolymers NR6 vary slightly, further supporting the conclusion of varying triad monomer sequences. See Figures 4.29 and 4.30 for 2D spectra attained.

Table 4.17. HSQC peak assignments for PSMA MW 350,000, 10-15%, mono-partial methyl ester, NR6.

<b><math>^1\text{H}</math> chemical shift /ppm</b>	<b><math>^{13}\text{C}</math> chemical shift/ppm</b>	<b>Correlated aliphatic assignments</b>
2.19-2.9ppm	32-43ppm	$\text{CH}_2$ - styrene
3.0-3.4ppm	38-44ppm	CH - maleic anhydride
6.6-7.7ppm	134-145ppm	$\text{C}_6\text{H}_5$ aromatic styrene

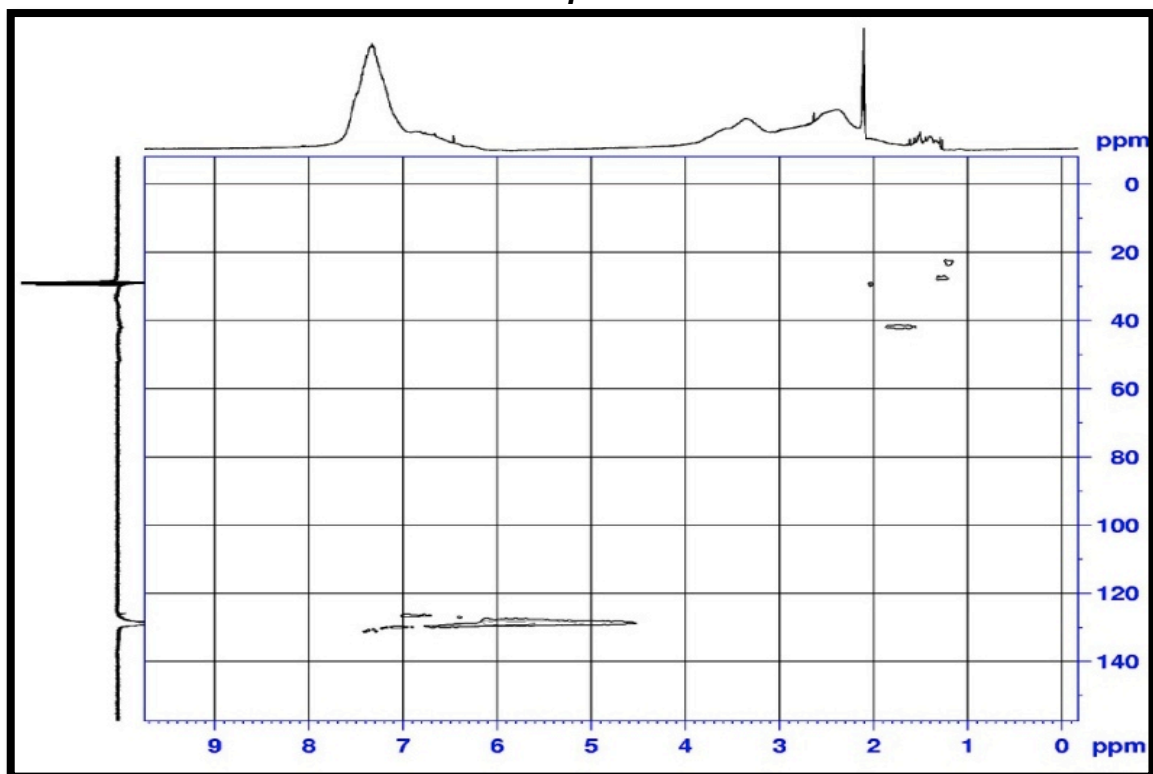


Figure 4.29. 2D HSQC spectra of PSMA MW 1,600 (1:1), NR1.

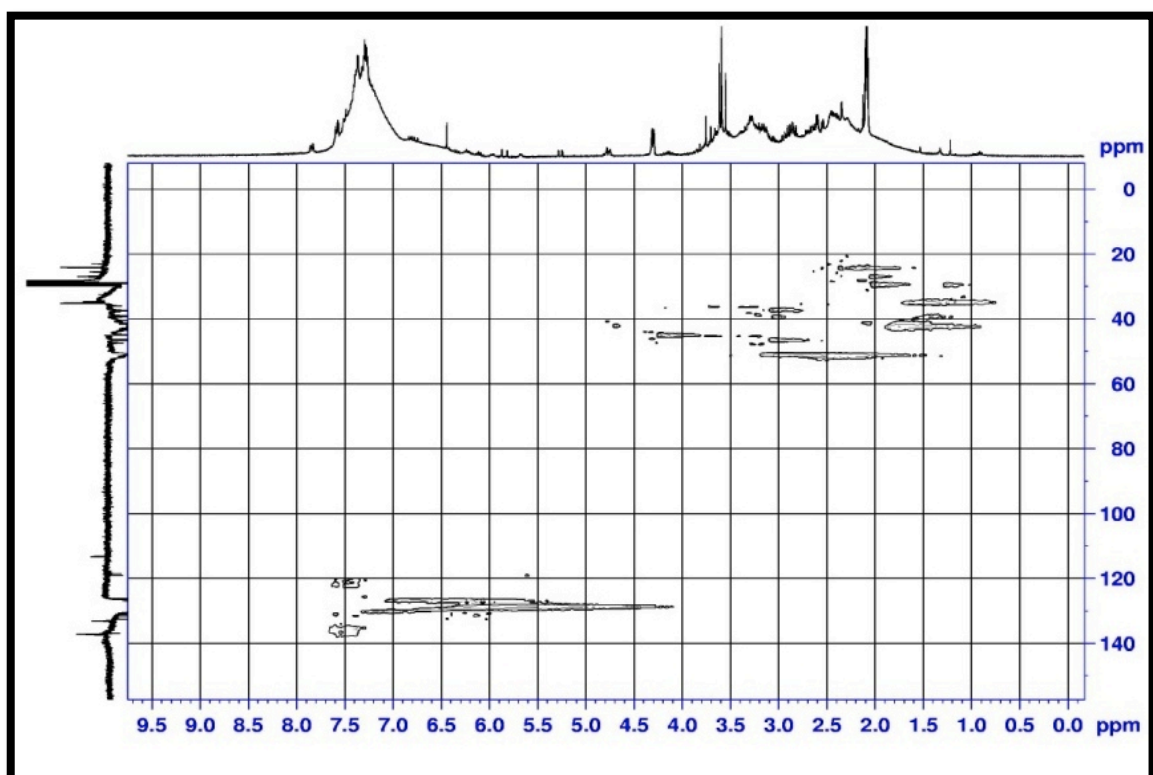


Figure 4.30 2D HSQC spectra of PSMA MW 350,000, 10-15%, mono-partial methyl ester, C-H correlation (NR6).

### **4.3 Discussion**

PSMA-based copolymers are known to undergo conformational transition in response to environmental stimuli. Due to the unique behaviour of PSMA-based copolymers, we have investigated the variations in structural properties that exist in commercially available PSMA-based copolymers. Although, the PSMA-based copolymers studied in this thesis were not synthesised for the application this work is concerned with the objective has been ultimately to further understand the structure-property relationships of PSMA-based copolymers. The physical behaviour of a polymer depends not only on the general chemical composition but also on the more subtle differences in microstructure, which are known to exist. Change in structure and microstructure will essentially modulate the pH-responsive behaviour.

Research findings for this study reveal that the chemical structure and microstructure for all PSMA-based copolymers are highly dependent on the synthesis methodology and conditions that the PSMA-based copolymers are produced and chemically modified. In terms of purity, the materials are free of any impurities, as no foreign matter has been identified from the  $^1\text{H}$ -NMR spectroscopy. However, those materials with a higher styrene feed ratio do exhibit more residual styrene monomer. PSMA-based copolymers should be carefully selected and designed for specific application requirements.

Evidentially, structural differences have been revealed from the analysis of the various types of PSMA-based copolymer NMR spectra. They all confirm and concur with the same general trends and findings.  $^1\text{H}$ -NMR spectra reports that the sequence distribution within a copolymer of styrene and maleic anhydride depends upon the monomer feed composition and the resulting copolymers can differ from 1:1 alternation. In the case where the molar ratio feed ratio of styrene-to-maleic anhydride is greater than 1:1 (for example 2:1, 3:1 or 4:1) an increasing number of styrene-styrene sequences are present. This results in a higher concentration of blocky (non-alternating triad) styrene monomer sequence arrangement.

Little information in the literature is available concerning the detailed structure and microstructure of PSMA-based copolymers. PSMA-based materials ideally need to be designed via careful polymerisation and modification techniques in order to satisfy the target specific structure and microstructure requirements. PSMA-based copolymers have been already utilised in important biomedical applications but the detailed

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structure-property relationships has not been previously investigated or discussed. This is important in order to design PSMA-based copolymers that are tailored to optimise performance, for purpose-made and more importantly for biomedical applications.

Despite the complexity and the challenge of analysing the  $^1\text{H}$ -NMR spectra acquired for all the PSMA-based materials, all NMR peaks have been successfully assigned. The differences in the styrene-to-maleic anhydride ratio are clearly visible from the spectral analysis. NMR analysis confirmed the presence of additional hydrophobic ester moieties (modified maleic anhydride moieties), as reported by manufacturers. Furthermore, it has been discovered that all the PSMA-based copolymers studied are not perfectly alternating.  $^1\text{H}$ -NMR also shows that the styrene content for the non 1:1 molar ratio PSMA-based copolymers appears lower than that reported by manufacturers. This provides evidence that discrepancies are apparent in the molar ratios reported for the PSMA-based copolymers by manufacturers and perhaps the molar ratios reported are the monomer feed ratio utilised during the copolymerisation reaction, as opposed to the actual final copolymer composition.

The most useful information was obtained from the edited  $^{13}\text{C}$ -NMR spectra (PENDANT) analysis. It confirmed that PSMA-based copolymers are indeed not perfectly alternating and more so for those materials with higher styrene content.  $^{13}\text{C}$  NMR studies have detected regioirregular structures, atactic and random monomer sequence distribution, giving rise to statistical PSMA-based copolymers, as opposed to regioregular structures, which would be expected for the reported 'so-called perfectly alternating' PSMA-based systems. However, as the PSMA systems microstructures seem to be of atactic in nature, this does not rule out alternating segments (triads) along the chain, which are likely to be in greater frequency for those PSMA-based materials with lower styrene content, i.e., for 1:1 styrene-to-maleic anhydride monomer feed ratio PSMA-based copolymers, e.g. in NR1.

The 2D NMR spectra confirmed the assignments for the  $^1\text{H}$ -NMR and edited  $^{13}\text{C}$  - PENDANT NMR spectra analysis, and further supported structures for the PSMA-based copolymers assigned. The spectra obtained clarified any peaks that were due to impurities such as solvent or residual monomer, i.e., matter that was not part (chemically bonded) to the PSMA-based copolymer structure.

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In conclusion, various NMR experiments have been carried out ( $^1\text{H}$ -NMR,  $^{13}\text{C}$ -NMR and 2D-HSQC NMR spectra) which confirm and provide concordant strong evidence that a broad range of PSMA-based copolymer structures are now commercially available for manipulation. All materials analysed were pure, with the higher styrene content materials exhibiting some residual monomer styrene. All PSMA-based materials did not demonstrate 100% perfectly alternating monomer sequence distribution. Those prepared from higher styrene monomer feed ratio conditions possessed higher styrene content and thus would have a higher concentration of 'blocky' styrene-to-styrene (non-alternating triad) monomer sequence distribution.

Overall, regioirregular structures, atactic and a random monomer sequence distributions have been detected for all the PSMA-based copolymers. On this basis, a more disordered monomer sequence distribution can be hypothesised for those materials with higher styrene content. Furthermore, this also predicts that a more ordered monomer sequence is expected for the 1:1 molar ratio systems and a higher concentration of alternating or semi-alternating triad monomer sequence distribution can be anticipated

The results presented here in Chapter 4 have shown strong evidence which enhances and clarifies our understanding regarding the structure-property relationships for PSMA-based copolymers, so that we can design novel lipid solubilising agents based from PSMA-based copolymers with precise and controlled structures which are effective at the target physiological conditions, satisfying the specific target structure requirements.

It is important to assess the surface chemistry of the novel PSMA-based structures sought in this study in order to determine which structure is the ideal effective lipid solubilising agent for the application this thesis is concerned with. Upcoming Chapter 5 studies the surface chemistry of the broad PSMA-based copolymers sought and relates the structural findings reported here to that concerning the surface behaviour of selected PSMA-based copolymers sought. Novel lipid solubilising agents are synthesised, static surface tension is measured via the du-Noüy method dynamic surface tension is recorded from Langmuir studies and the frictional behaviour of some interesting lipid solubilising agents is also studied.

**CHAPTER 5:  
THE SURFACE CHEMISTRY OF  
NOVEL PHOSPHOLIPID  
SOLUBILISING AGENTS (STYRENE-  
MALEIC ACID BASED  
COPOLYMERS) AS SYNTHETIC  
PROTEIN ANALOGUES**

## **5.0 Introduction**

This chapter discusses the methodologies developed and reports the observations recorded during the hydrolysis of a broad range of PSMA-based copolymer structures sought in Chapter 4, whilst synthesising novel polyanionic surfactants (lipid solubilising agents). The manipulation of the maleic anhydride functional group is discussed in section 5.1. Additionally, the importance and complexity of the fundamental carboxylic acid chemistry, which is essentially the 'pH responsive moiety switch', is studied. The relationship between the isoelectric point (IEP), critical pH ( $pH^*$ ), hydrophilic-lipophobic balance (HLB) and the  $pK_a$  of the broad range of novel lipid solubilising agents prepared is summarised. This in turn, has allowed definition for the target properties for an ideal polar phospholipid solubilising agent, in particular for boundary biolubrication applications. Finally, section 5.3 deals with assessing the *in-vitro* surface characterisation of some interesting synthetic surfactant protein analogues (lipid solubilising agents) developed.

## **5.1 Hydrolysis of PSMA-based copolymers**

The PSMA-based copolymer under study (if in solid form) was ground in a pestle and mortar to generate a fine powder. It was then hydrated in HPLC grade water (into 80% of the total required volume) and left to stir on a magnetic stirrer, until a homogeneous solution was achieved. 1M sodium hydroxide solution was then added (20% of the total required volume) in a dropwise manner until a clear solution was obtained, which was above the  $pK_a$  value of the acid functionality. When a clear solution was obtained the remaining 100% volume was made up with the HPLC grade water, respectively. For some PSMA-based solutions, it was necessary to heat them to  $>50^{\circ}C$  to optimise the hydrolysis of the maleic anhydride groups to maleic acid. The solution was stirred over a period of 48 hours and then refrigerated. The ease of the hydrolysis was dependent on the PSMA-based copolymer structure type. The heating of the solution was necessary for some copolymers. The hydrolysed PSMA-based solutions were then stored in a fridge until utilised. The prepared polyacids (lipid solubilising agents) were also successfully freeze dried in the same method, as described in Chapter 3. From all the PSMA-based copolymers studied, NR6 was the easiest to hydrate, despite the fact that it possessed the highest molecular weight. It is interesting to note that this PSMA copolymer also has additional hydrophobic mono-partial methyl ester functionality. This was available from S-A - see Table 5.1 for further details. NR6 also dissolved into HPLC grade water upon hydrolysis at a pH of around 6, in contrast to



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most other PSMA-based copolymers that dissolved at around  $>\text{pH } 9$ . Even at this stage, NR6 behaved in a unique manner, in comparison to all other PSMA-based copolymers. The mono-methyl partial ester available from SAR (see Table 5.1) was also easily hydrolysed. The more hydrophobic PSMA-based copolymers (NR2, NR4, NR11, and NR12) available from POL and SAR (with higher styrene content) required heating to  $>50^{\circ}\text{C}$ , for at least 48 hours to dissolve in alkaline solution.

The salt solutions; NR4, NR8 and NR14 available from and SAR and S-A (see Table 5.1) respectively, were easily diluted to the required concentration. PSMA-based copolymers (NR1 and NR14) available from S-A and SAR, i.e., those that are marked with a 1:1 monomer feed ratio of styrene-to-maleic anhydride, dissolved in alkaline solution without heating, but at a pH of around 10.

In conclusion, overall all PSMA-based copolymers summarised in Table 5.1, were successfully hydrolysed at  $>50^{\circ}\text{C}$  (in alkaline conditions). Furthermore, it was also interesting to note that they were also successfully hydrolysed in phosphate buffer 7 solutions too.

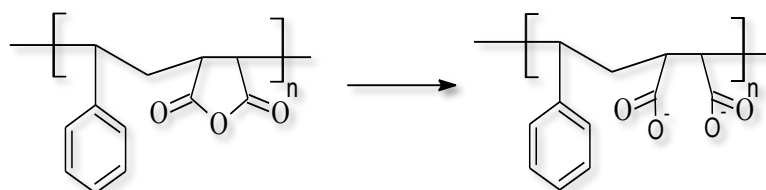


Figure 5.1. Hydrolysis of full anhydride PSMA-based copolymers

Table 5.1, summarises the observation noted for some interesting PSMA-based copolymers hydrolysed to form novel phospholipid lipid solubilising agents possessing various polymer compositions and molecular weight.

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Table 5.1. A summary of the observations noted during the hydrolysis of the various PSMA-based copolymers studied from POL, S-A and SAR, respectively.

<i>Manufacturer/MW/code</i>	<i>pH on clearing</i>	<i>clear at &gt; 50°C</i>
	<i>25°C (24 hours)</i>	<i>(48 hours)</i>
(P) / 1, 600 (NR1)	9.6	yes
(P) / 1, 900 (NR2)	10	<10/yes
(P) / 7, 500 (1:2.3) (NR4)	No	<10/yes
(P) / 9, 500 (3:1) (NR3)	No	<10/yes
(SAR) / SMA® 1000P (NR10)	No	<10/yes
(SAR) / SMA® 2000P (NR11)	9	<10/yes
(SAR) / SMA® 3000P (NR12)	9	<10/yes
(S-A) / 350, 000 (NR6) - partial ester	5.6-yes	yes
(S-A) / 1, 900 (NR7) - partial ester	9	<10/yes
(S)/ SMA® 17352 (NR13) - partial ester	8	<10/yes
(S-A) / 180, 000 (NR9) - partial ester	9	<10/yes
(S-A) / MW120, 000 - 30% wt./v (NR5)	PSMA hydrolysed solutions were diluted with water, to obtain desired PSMA % (w/v) solution.	
(S-A) / MW350, 000 - 13% wt/v (NR8)		
(SAR)/MW1000NA - 40% wt/v (NR14)		

In the hydrolysed form, PSMA has two acid groups. One acid group can be linked with four water molecules by hydrogen bonding. Therefore, eight water molecules will be necessary to describe the close hydrophilic interactions between the PSMA molecules and the solvent molecules. Indeed, these interactions imply hydrogen bonding and therefore electronic exchange between the solute and solvent. The carboxylic pendant groups accept protons at low pH, whilst releasing them at high pH. Therefore, they are transformed into polyelectrolytes at high pH, with electrostatic repulsion forces, between the molecular chains. An acid dissociation constant,  $K_a$ , (also known as acidity constant, or acid-ionization constant) is a quantitative measure of the strength of an acid in solution. It is the equilibrium constant for a chemical reaction known as dissociation in the context of acid-base reactions, or many practical purposes it is more convenient to discuss the logarithmic constant,  $pK_a$ . As the environmental pH changes, the degree of ionisation in a polymer bearing weakly ionisable groups is dramatically altered at a specific pH (i.e., the  $pK_a$ ). The rapid change in net charge of pendant groups causes an alternation of the hydrodynamic volume of the polymer chains. The transition from collapsed state to expanded state is explained by the osmotic pressure exerted, by mobile counterions neutralising the network charges. Hydrolysed PSMA-

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based copolymers are weak polyacids; the carboxylic group moiety being the weak acid functionality. It is therefore important to understand the complex chemistry of the 'pH responsive moiety switch'. Since hydrolysed PSMA-based copolymers are weak acids, the following equilibrium exists in the aqueous solution of PSMA-based copolymers [43-44, 106-112].

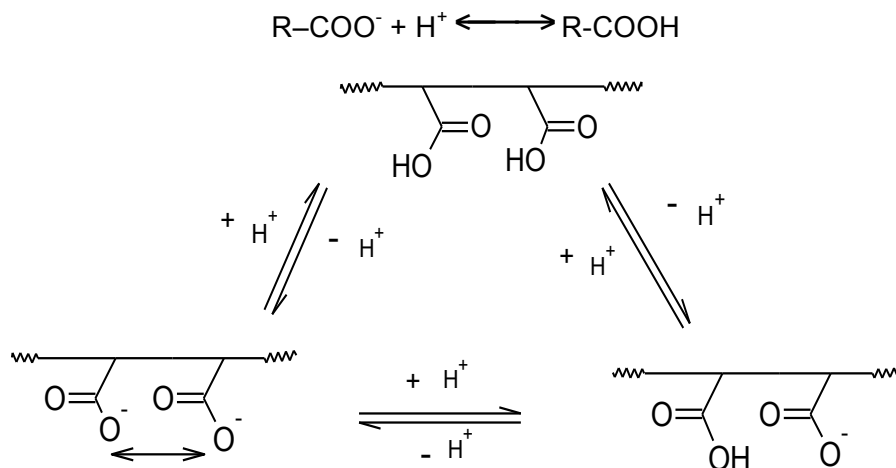


Figure 5.2. Possible structures of hydrolysed poly(maleic acid), at different pH values.

Therefore,  $\text{RCOO}^-$  and  $\text{RCOOH}$  coexist in the solution, and their relative concentrations will vary with pH. It is expected that a decrease in the solution pH, will increase the concentration of  $\text{R-COOH}$ . The carboxylic group ( $\text{COOH}$ ) is one of the most widely occurring functional groups in chemistry and biochemistry. Carboxylic acids are polar substances. The functional group can form strong hydrogen bonds with each other and with water. Copolymers of styrene and maleic anhydride (1:1 molar ratio) (i.e., hydrolysed styrene/maleic anhydride polymers) have a  $\text{pK}_a$  value in region of 3.75 - 4. The  $\text{pK}_a$  for the individual acid functions are summarised below in Table 5.2.

Table 5.2. Dissociation properties of maleic acid [2].

Compound	Equilibrium	$\text{pK}_a$	$\Delta H^\theta / \text{kJmol}^{-1}$	$T\Delta S^\theta / \text{Jmol}^{-1}$
maleic acid	$\text{H}_2\text{A} \rightleftharpoons \text{HA}^- + \text{H}^+$	1.92	1.10	9.85
maleic acid	$\text{HA}^- \rightleftharpoons \text{H}^+ + \text{A}^{2-}$	6.27	-3.60	39.4

The acid base chemistry of the maleic acid functionality in PSMA-based copolymers is very complicated and thus deserves a thorough discussion. Maleic acid has  $\text{pK}_a$  values separated by several orders of hydrogen ion concentration. The cis isomer ionises in two steps. After the first ionisation, intramolecular hydrogen bonding stabilises the cis monoanion. One of the carboxylic groups deprotonates, the other carboxylic group can form a strong hydrogen bond to it; overall, the effect is to favour

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the deprotonated state of the hydrogen-bond-accepting group (lowering its  $pK_a$  from  $\sim 4$  to 1.92) and to favour the protonated state of the hydrogen-bond-donating group (raising its  $pK_a$  from  $\sim 4$  to 6.07). As a result the first ionisation is easier, and the second is difficult. The two-step dissociation behaviour can be clearly displayed for the dicarboxylic acid only when the two acid groups are in close enough proximity to exert influence on each other. In other words, the ionisation of the first carboxylic acid group can impose steric and electrostatic hinderance on the ionisation of the second carboxylic acid group when the two acids are in close proximity. The acidity of the first carboxylic acid is stronger due to the inductive effect of the adjacent carboxylic acid group. The presence of neighbouring dipoles promotes the ionisation of carboxylic acid groups. The second carboxylic acid group is much less acidic, due to the repulsive effect of the ionised adjacent group and to the stabilisation of the monoanion by an intramolecular hydrogen bond.

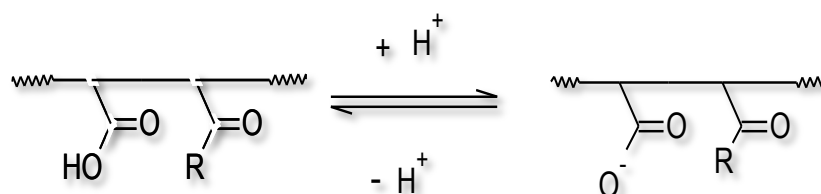
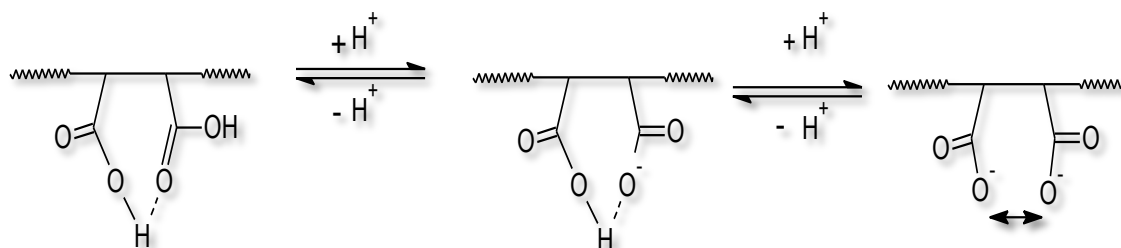


Figure 5.3. Possible structures of mono-partial ester hydrolysed-based copolymers.



Relative weak hydrogen bond  
(carboxylate as donor)

Ideal hydrogen bond

destabilised by dipole-dipole interactions

Figure 5.4. Dissociation of poly(maleic acid), full anhydride converted-based copolymers and hydrogen bonding.

When a full anhydride (unmodified) based copolymer is employed for formation of a polyacid (see Figure 5.4 for mechanism and structures), intramolecular hydrogen bonding takes place at the expense of intermolecular interactions. The two carboxyl groups in the molecule of the cis isomer maleic acid form an intramolecular hydrogen bond. As a result, there will be fewer carboxyl groups available for the formation of intermolecular hydrogen bonds. Only one intermolecular hydrogen bond per molecule is formed. Furthermore, in the case where a mono-partial ester PSMA is employed to form a polyacid (see Figure 5.5), the presence of the ester group hinders the overall

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intramolecular hydrogen bonding, although the carboxyl charged groups will be available for intermolecular hydrogen bonding [43-44106-112].

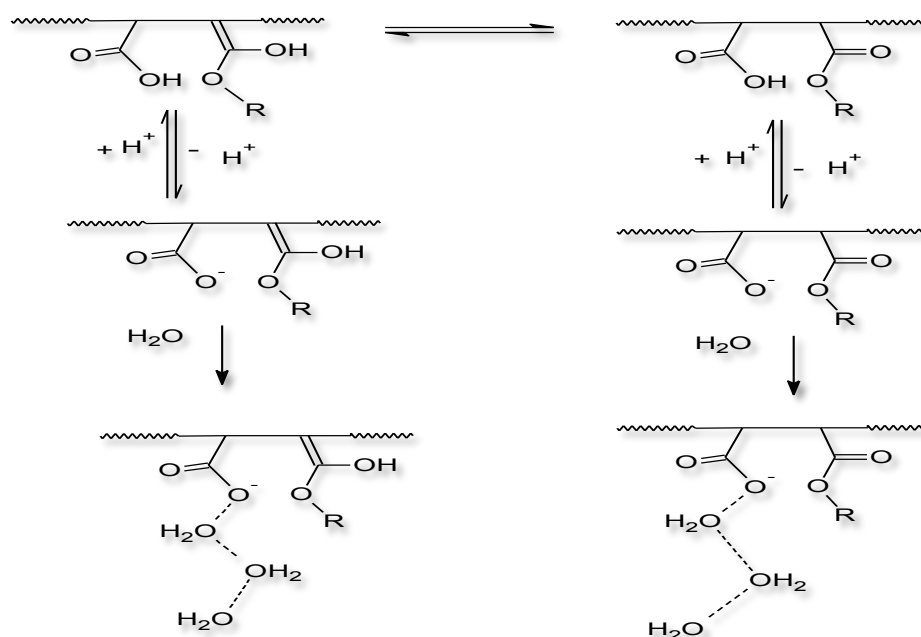


Figure 5.5. Dissociation of mono-partial ester based PSMA-based copolymers and hydrogen bonding with solute in the absence of intramolecular hydrogen bonding.

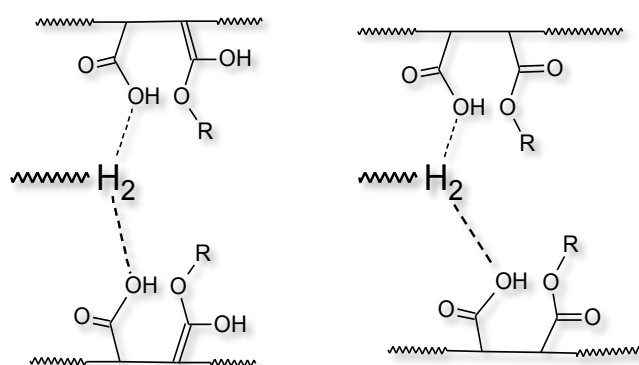


Figure 5.6. Possible intermolecular hydrogen bonding of mono-partial ester functionality.

At 50% protonation, internal hydrogen bonding form between the COO<sup>-</sup> and COOH groups of succinic acids, which compose 50% of the polymer structure for 1:1 molar ratio PSMA-based copolymers. This stiffens the polymer backbone, making it linear. These hydrogen bonds are weakened at increased protonation and deprotonation. The mono-partial ester PSMA types have segments along the PSMA chain, where there is only one acid group per anhydride hydrolysed moiety. This will obviously have impact on the overall nature and strength of hydrogen bonding; the stabilising force for macromolecules during the self-assembly, in aqueous media. The carboxylic groups can dissociate, giving rise to charges on the macromolecules chain, so that the copolymers behave in aqueous solution as polyelectrolytes. Polyelectrolytes are water

soluble polymers that carry ionised or ionisable groups. In aqueous solution they are dissolved into macroions (polyions) and ions of opposite charge - counterions. Electrostatic repulsion between adjacent carboxylic groups of maleic acid units exists, and hydrophobic interactions between non-polar segments may be present. The dissociation of maleic acid copolymers is the main factor responsible for the polyelectrolyte behaviour in aqueous solution [43-44, 106-112].

### **5.1.1 Modulating the critical pH ( $pH^*$ ) of lipid solubilising agents**

The main aim of this study was to design PSMA-based copolymers that mimic the functionality of native apoproteins (surfactant protein analogues), in solubilising polar phospholipids (boundary lubricant agents) into aqueous media, at the target pH (physiological pH). Essentially, the target critical pH for the ideal lipid solubilising agents would be at physiological pH. The challenge is therefore to achieve a balance of good hydration (sufficient hydrophilic moieties) and additionally to adjust the  $pK_a$  such that the critical  $pH^*$  approaches physiological pH. This is governed by achieving an appropriate balance of hydrophobic association and electrostatic repulsion. Incorporating a hydrophobic moiety might increase the  $pH^*$ . The pH range, over which a reversible phase transition occurs, can be generally modulated by two strategies; selecting the ionisable moiety with a  $pK_a$  matching the desired pH range, and changing the critical pH ( $pH^*$ ) range by incorporating a hydrophobic moiety into the polymer backbone. The  $pK_a$ , at which half of the ionisable are ionised, is much related to  $pH^*$ . Adjusting the appropriate  $pH^*$ , at which reversible conformational changes (usually followed by phase transitions) of polymer chains occur, is an important factor for the pH-responsive polymer-based application [27-35]. The intrinsic  $pK_a$  values of an ionisable moiety should be adopted as a first consideration in the selection of a proper pH-responsive polymer for the desired application. The  $pK_a$  for each polyacid prepared in this thesis will be dependent on the HLB value of the PSMA-based copolymer. So by preparing polyanionic surfactants from more hydrophobic based PSMA copolymers, i.e., those that have a higher hydrophobic styrene/partial esters moieties content, weaker polyacids are prepared and the  $pH^*$  of the novel lipid solubilising agent/polyanionic surfactant is higher. Introducing a more hydrophobic moiety can offer a more compact conformation in the uncharged state and a more dramatically discontinuous phase [109]. S.Mafe[106] reports that the  $pK_a$  values of ionisable groups in macromolecules can be significantly different than those of the isolated groups in solution because of interactions between neighbouring ionisable groups, solvation effects, and conformational changes. The electrostatic interactions between negative

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and positive fixed charge groups are responsible for many of the observed phenomena in amphoteric surfaces. Electrostatic interactions play also a key role in the determination of the  $pK_a$  values of the acid and basic residues that control the permeability of ion channels to charged solutes [27-35, 106, 113-119].

### **5.1.2 The relationship between HLB, $pK_a$ , IEP and $pH^*$**

Essentially, the hydrophobic-lipophobic (HLB), of a surfactant is a measure of the degree to which it is hydrophilic or lipophilic, determined by calculating values for the different regions of the molecule, as described by Griffin in 1949 [92]. The isoelectric point IEP - the pH at which a particular molecule carries no net electrical charge and the  $pK_a$  (the logarithmic acid dissociation constant) of hydrolysed PSMA-based copolymer are interrelated and thus dependent on each other. Although, these properties can be determined independently, they are related, and are a consequence of the microstructure of the starting PSMA-based copolymer (the styrene-to-maleic anhydride/partial ester functionality) and the methodology employed to hydrolyse and modify the maleic anhydride functionality. This is essentially the method by which the polyanionic surfactants (novel lipid solubilising agents) are prepared. The balance between the hydrophilic and hydrophobic segments of a surface active agent (surfactant) can cover a wide range; to molecules that are almost completely insoluble in water to ones that are highly soluble. The amphiphilic nature of many emulsifying agents (particularly non-ionic surfactants) can be expressed in terms of an empirical scale of so called HLB (hydrophile-lipophile balance) numbers. The HLB system was designed for non-ionic surfactants and typically ranges from 1 (most hydrophobic) to 20 (most hydrophilic). The ranges of HLB values for the different types of nonionic surfactants, based on their water solubility is generally defined as follows: Griffin [92] devised an arbitrary scale of values to serve as a measure of the hydrophilic and-lipophilic balance (HLB) of surface active agents. By means of this number system, it is possible to establish a HLB range of optimum efficiency for each class of surfactant. The target HLB for an ideal lipid solubilising agent would in theory, be between 13-18, according to the Griffin scale (see table 5.3) [92].

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Table 5.3. HLB values and their applications [92].

<b><i>Applications</i></b>	<b><i>Dispersibility in water</i></b>
3-6 W/O emulsions	1-4 nil
7-9 wetting agents	3-6 poor
8-15 O/W emulsion	6-8 unstable milky dispersion
13-15 detergent	8-10 stable milky dispersion
15-18 solubiliser	10-13 translucent dispersion/solution
	13 clear solution

### **5.1.3 Estimation of HLB values of polyanionic surfactants synthesised**

Anionic surfactants, like hydrolysed PSMA-based solutions, generally have a much higher HLB value than nonionics due to the higher hydrophilicity of the anionic group (maleic acid). For the styrene-maleic anhydride polymers, the multiplier may need to be smaller based on the fact that they are polymeric surfactants, which lowers their ability to migrate to interfaces compared to the monomeric surfactants. If we take into consideration the applications of the styrene-maleic anhydride copolymers with, it would appear that the appropriate multiplier for the styrene-maleic anhydride copolymers might be about 2.8. This value was taken by the literature reported by the PSMA copolymer manufacturer Sartomer (SAR). This method, as reported by SAR (a manufacture of PSMA-based copolymers), has been used for all the different PSMA-based copolymers studied in this thesis. Using this assumption, the range of hydrolysed PSMA-based copolymer would have the approximate values listed in Table 5.4.

HLB values are traditionally calculated for nonionic surfactants and can be determined experimentally by trying to emulsify liquids of known HLB value. The values of the anionic PSMA/esters have been calculated using the nonionic method. A correction factor has been applied to adjust them into a range where they appear to be consistent with the known applications. The more hydrophilic materials contain more maleic anhydride and/or acid groups and have the higher HLB values. The products that have more styrene and longer ester chains are more hydrophobic and thus have lower HLB values. Although, the values shown in the above Table 5.4 may not be exact, the trend seems to be consistent with the structures and known behaviour. For example the SAR-SMA<sup>®</sup>1000P based copolymer (NR10) has a  $M_n = 2000$  and an acid number of 480mg KOH/gm., i.e., 48% maleic anhydride content. Therefore, by the nonionic HLB



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method, we would divide this percentage figure by 5 and get the HLB as, 9.6. Using the theoretical 2.8 multiplier method adopted by SAR, the HLB value for the SAR SMA®1000P is 26.88. For the partial esters, the alkyl portion of the ester was counted into the hydrophobic portion of the molecule. The same methodology was used to calculate the HLB value for all the PSMA hydrolysed studied in this thesis.

Table 5.4. A summary of the estimated HLB values for all hydrolysed PSMA-based copolymers studied. The items underlined are PSMA-based solutions that possess suitable properties for surfactant protein analogues.

Hydrolysed	MW	Hydrophilic	HLB value
PSMA code		component	(surfactant class)
Polysciences (POL)			
NR1	1, 600	50%	28 (most hydrophilic)
NR2	1, 900	50%	28 (most hydrophilic)
<u>NR3</u>	<u>7, 500</u>	<u>33%</u>	<u>14 (detergent)</u>
<u>NR4</u>	<u>9, 500</u>	<u>25%</u>	<u>18.48 (solubiliser)</u>
Sigma-Aldrich (S-A)			
NR5	120, 000	50%	28
<u>NR6</u>	<u>350, 000</u>	<u>35-40%</u>	<u>17.5 (detergent/solubiliser)</u>
NR7	1, 900	11%	6.16 (most hydrophobic) (O/W-emulsifying agent)
NR8	350, 000	50%	
NR9	180, 000	35-40%	10.4 (W/O-emulsifying agent)
Sartomer (SAR)			
NR10	5, 500	48%	26.7
<u>NR11</u>	<u>7, 500</u>	<u>27%</u>	<u>15.1 (detergent)</u>
<u>NR12</u>	<u>9, 500</u>	<u>33.5%</u>	<u>19.88 (solubiliser)</u>
<u>NR13</u>	<u>7, 000</u>	<u>27%</u>	<u>15.2 (detergent)</u>
NR14	5, 500	50%	28 (most hydrophilic)

From all the PSMA-based copolymers studied (with respect to the estimated HLB values), several trends are evident:

The most hydrophilic hydrolysed PSMA-based copolymers were prepared from those with the highest estimated HLB values - the 1:1 monomer feed ratio styrene-to-maleic

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anhydride copolymers (HLB = 26-28), corresponding to NR1, NR2, NR5 and NR14, see Table 5.4 and Table 5.1 for further details.

The most hydrophobic PSMA-based copolymers were attained from NR7 and NR9 with a (HLB = 6.16 and 10.4), - see Table 5.4 and Table 5.1 for further details. Generally, the polyanionic surfactants with a greater concentration of hydrophilic charged maleic acid groups, achieved good solubility in water upon hydrolysis, due to greater hydrogen bonding effects.

The ideal objective described for this work was to seek 'surfactant' protein analogues (as effective lipid solubilising agents) for boundary lubrication applications, at the physiological pH. This is because one of the requirements of a boundary lubricant is that it must have the ability to adsorb at the biological interface. This behaviour is typical of surfactants (surface active agents).

Section 5.2, discusses how these HLB values for surfactant protein analogues correlate well with numerical surface measurements obtained. The hydrolysed PSMA-based copolymers that fall into this category are; NR3, NR4, NR6 and NR11 and NR13, respectively, and are underlined in Table 5.4.

Of all the PSMA-based copolymers studied, NR3, NR6, NR11 and NR13, in theory, seem ideal candidates as lipid solubilising agents for boundary biolubrication applications. The HLB scale generated (see Table 5.4) gives a relative comparison for the optimum surfactant efficiency for each hydrolysed PSMA developed. A higher HLB value relates to a more hydrophilic polyanionic surfactant, and a lower HLB value thus a more hydrophobic polyanionic surfactant.

The data listed in Table 5.4, indicates that a broad range of PSMA-based copolymers, are now available (which extends their applications) and allows for a greater selection for tailor-made pH-responsive behaviour. The information determined in this study, is a good starting point to predict the behaviour of hydrolysed PSMA-based copolymers and thus is useful when selecting and designing PSMA-based copolymers for specific application requirements.

## **5.2 Characterisation of synthetic surfactant protein analogues**

The in-vitro surface properties of the polyanionic surfactants synthesised are investigated in this study. The requirement for an effective lipid solubilising agent in boundary lubrication applications (e.g., in ocular lubrication applications) is that it must be surface active, at the air-aqueous interface. It is therefore important to determine which PSMA-based materials (prepared in this study) fulfil this criterion most effectively. Many compounds have hydrophilic and hydrophobic regions. Because of this dual structure, it is energetically favourable for these materials, when dissolved, to adsorb at interfaces, orientating themselves in such a manner that the regions are associated with the appropriate solvent or air. Such materials are termed surface active agents and are said to be amphipathic; i.e., they possess both hydrophilic and hydrophobic moieties that orientate themselves according to the nature of the interface they are exposed to.

### **5.2.1 Interfacial phenomena: surface chemistry**

When phases exist together, the boundary between two of them is called an interface. Matter at interface usually has different characteristics from that in the bulk of the media, and as a consequence the study of interfaces has developed into a separate branch of chemistry; surface chemistry. Interfaces are categorised according to the phases they separate, as follows: liquid/liquid (L/L), liquid/vapour (L/V), solid/vapour (S/V) and solid/liquid (S/L). It is convenient to treat each interface separately. Interfaces are everywhere. An important example concerns the inner lining of the lung. A fluid lines the alveoli, and at the surface of this fluid, in contact with the air, is a layer that is only one molecular thick, which is composed of phospholipids. This mixture of phospholipids and some proteins is known as lung surfactant. Lung surfactant serves to lower the amount of work required for the action of breathing, a function that is so important that its absence makes unaided breathing impossible. Many commonly occurring phenomena that are observed in systems containing an interface in which one of the phases is a liquid can be understood through the concept of surface tension [120-125].

### **5.2.2 Surface measurements: measuring surface tension**

The surface tension is a fundamental quantity in the investigation of fluid interfaces and so its measurement is of great importance. Many methods exist for measuring the value of the surface tension of an interface, and the choice of method depends on the given system. The surface tension of the interface between a static, aqueous solution and air can be measured by a number of methods. The surface tension is the force per unit length, acting on an imaginary line drawn in the surface and has SI units of mN/m.

Molecules in the bulk of the liquid are surrounded in all directions by other molecules for which they have an equal attraction [120-125]. On the other hand, molecules at the surface (i.e., at the liquid-air interface) can only develop attractive cohesive forces with other liquid molecules that are situated below and adjacent to them. They can develop adhesion forces of attraction with the molecules constituting the other phase involved in the interface; this adhesive force of attraction is small. The net effect is that the molecules at the surface of the liquid experience an inward force towards the bulk. Such a force pulls the molecules together and, as result, contracts the surface, resulting in a surface tension [120-125].

Surface tension - and the more fundamental quantity, surface free energy - fulfil an outstanding role in physical chemistry of surfaces. The surface tension of a liquid is often defined as the force acting at right angles to any line of unit length on the liquid surface. However, this definition (although appropriate in the case of liquid films, such as in foams) is somewhat misleading, since there is no elastic skin or tangential forces as such at the surface of a pure liquid. It is more satisfactory to define surface tension and the surface free energy as the work required to increase the area of a surface isothermally and reversibly by unit amount. At the interface between two liquids there is an imbalance of intermolecular forces but to a lesser magnitude. Interfacial tensions usually lie between the individual surface tensions of the two liquids in question. The short-range intermolecular forces which are responsible for surface/interfacial tension include Van der Waals forces (in particular, London dispersion forces, which is universal) and may include hydrogen bonding and metal bonding. There are several methods available for the measurement of surface tension. The Wilhelmy plate and the du Noüy sensitometer method were both employed in this study [91-92,120-125].

### **5.2.3 Measuring static surface tension via du-Noüy ring method**

The surface tensions of hydrolysed PSMA-based solutions were measured using a Pt-Ir, du Noüy ring. All measurements were recorded to the nearest 0.1mN/m at room temperature. The static surface tension was measured at least three times for each sample, to obtain the mean static surface tension. To assess the viability of the data the standard deviation was also calculated. All glassware was cleaned with commercial Fairy Liquid and left to rinse under hot tap water for 15 minutes. The Pt-Ir, du-Noüy ring was rinsed with HPLC grade water and then flamed prior to being placed on the microbalance for sample measurements. Before each sample was measured the static surface tension of HPLC grade water was measured (approximately 72mN/m). The hydrolysed PSMA-based solutions were allowed to rest before measurements were undertaken, to avoid any foam formation. Three different molecular weights of 3% (w/v) hydrolysed PSMA (MWs 1, 600, 1, 900 and 350, 000 respectively) were hydrolysed in aqueous solution, under alkaline conditions (above pH 7). The hydrophilic and hydrophobic balance was also varying due to the variation in styrene-to-maleic anhydride monomer feed ratio and the partial propyl and methyl ester functionality respectively.

Table 5.5. Properties of PSMA-based hydrolysed solutions - used in static surface tension measurements.

<b><i>MW/code</i></b>	<b><i>MA:St monomer feed ratio</i></b>	<b><i>Modification</i></b>	<b><i>HLB</i></b>	<b><i>Acid number</i></b>
1, 600 (NR1)	1:1	none	28	480 KOH/gm
350, 000 (NR6)	1:1	partial methyl ester	17.5	unknown
1, 900 (NR 7)	2:1	partial propyl ester	6.1	200-240 KOH/gm

A solution of 3 % solution was used as this was the optimum concentration at which the polymer solution generated a clear solution with sufficient viscosity for lubrication applications. Furthermore, this was also the maximum concentration that provided effective phospholipid (DLPC/DPPC) complexation.

The solutions were heated at 50°C for at least 48 hours, to ensure complete hydrolysis of the maleic anhydride functionality to maleic acid, in order to achieve a higher degree % ionisation required for optimum surfactant behaviour.

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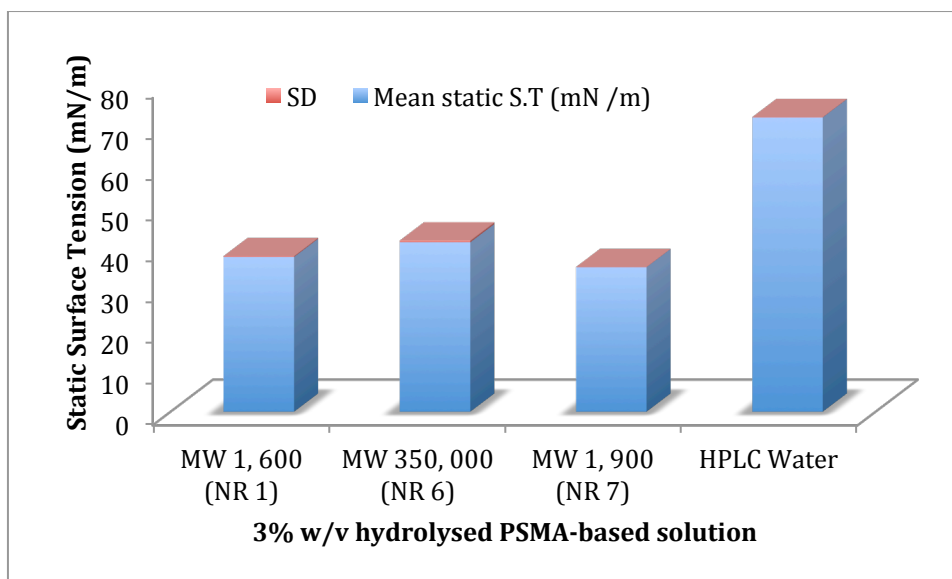


Figure 5.7 Average static surface tension measurements of 3% (w/v) PSMA solutions.

Table 5.6. The average static surface tension of 3% (w/v) PSMA-based hydrolysed solutions

3% (w/v) PSMA/code	Mean static S.T	SD	HLB
MW 1, 600 (NR 1)	37.8 mN /m	0.22	28
MW 350, 000 (NR 6)	41.4 mN /m	0.42	17.5
MW 1, 900 (NR 7)	35.3 mN /m	0.05	6.16
HPLC Water	71.9 mN /m	0.08	-----

Figure 5.8, shows the static surface tension measurement of HPLC grade water by the du Noüy ring method. The method involved slowly lifting a platinum ring, from the surface of a liquid. The force (F), in mass mg is plotted against the distance travelled (mm). Water has a high surface tension, so the force required to raise the ring at the surface of water was high as around 600 mass mg. The force (F) required to raise the ring from the liquid's surface is measured and related to the liquid's surface tension. This type of tensiometer uses a platinum ring which is submersed in a liquid. As the ring is pulled out of the liquid, the force required is precisely measured in order to determine the surface tension of the liquid. This method requires that the platinum ring be nearly perfect; even a small blemish or scratch can greatly alter the accuracy of the results. A correction for buoyancy must be made. This method is considered less accurate than the plate method but is still widely used for interfacial tension measurement between two liquids. See section 3.8.1 (Chapter 3, page 69) for full details of the experimental methods employed and Figure 3.9 (chapter 3, page 72) for explanation of the plots presented in Figure 5.8-5.11.

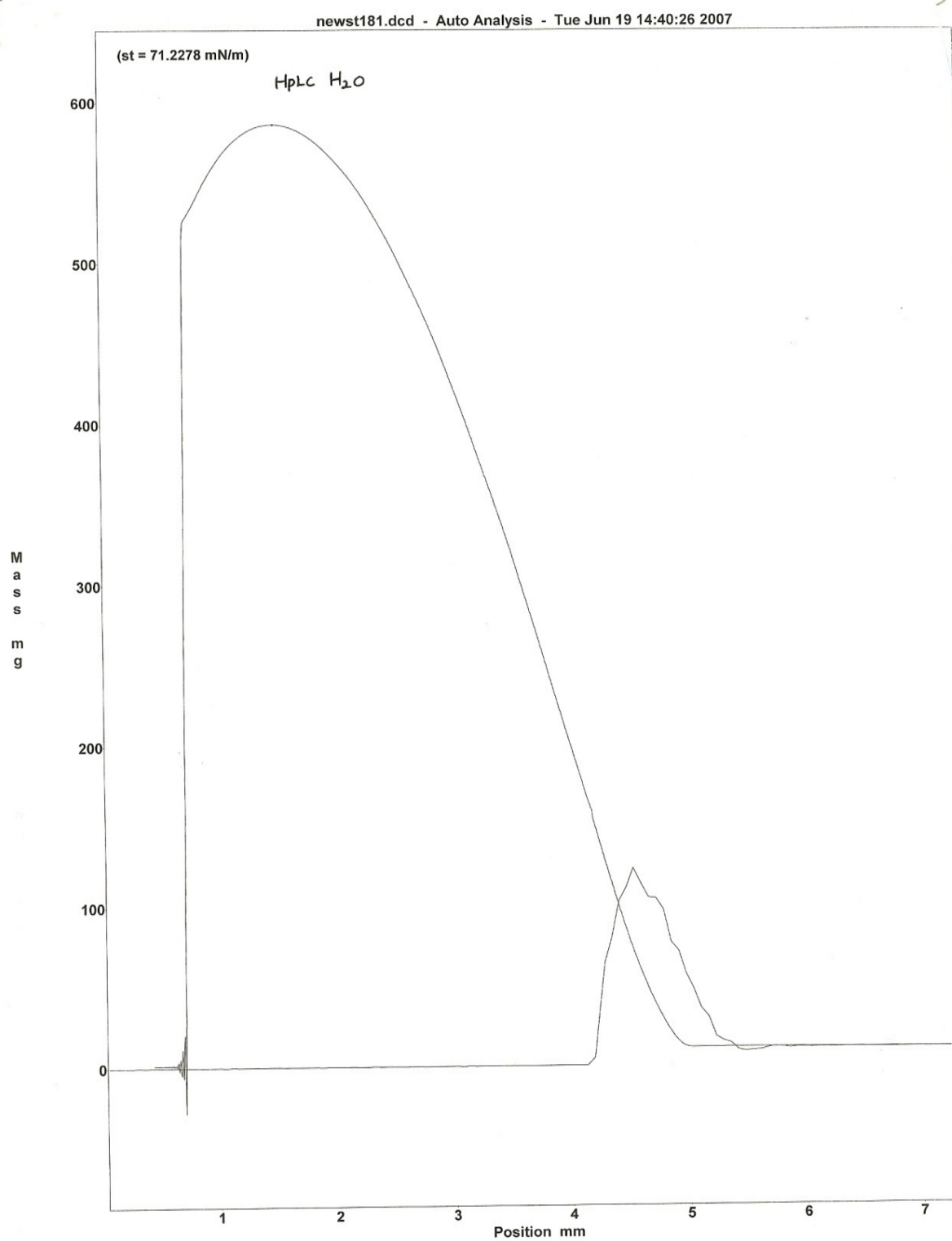


Figure 5.8. The static surface tension of HPLC water - 71.3mN/m (control).

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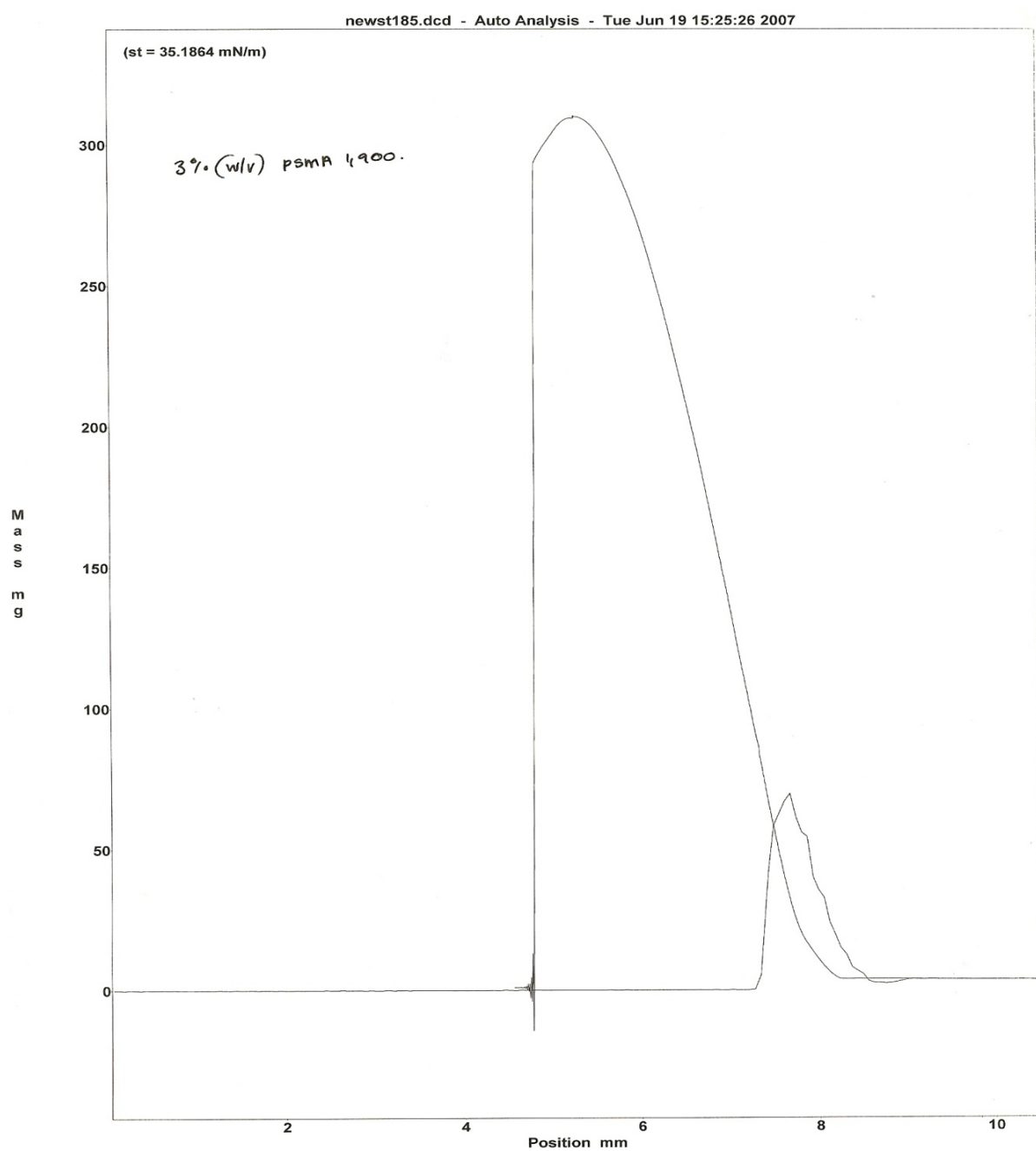


Figure 5.9. The static surface tension (35.2mN/m) measurement of 3% (w/v) PSMA MW 1,900 - partial propyl ester, at room temperature.



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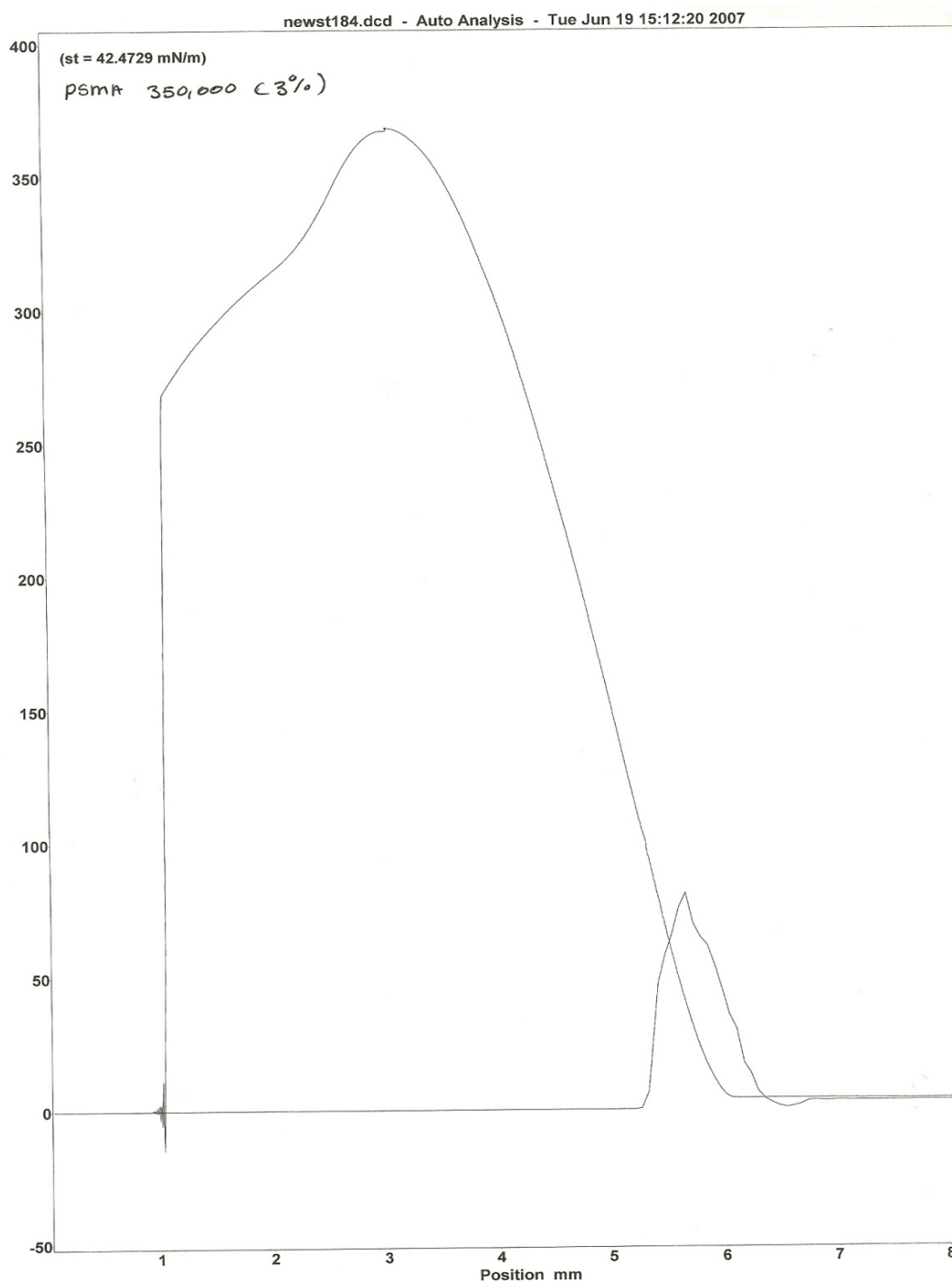


Figure 5.10. The static surface tension (42.5mN/m) of 3% (w/v) PSMA MW 350, 000 - mono-partial methyl ester solution.

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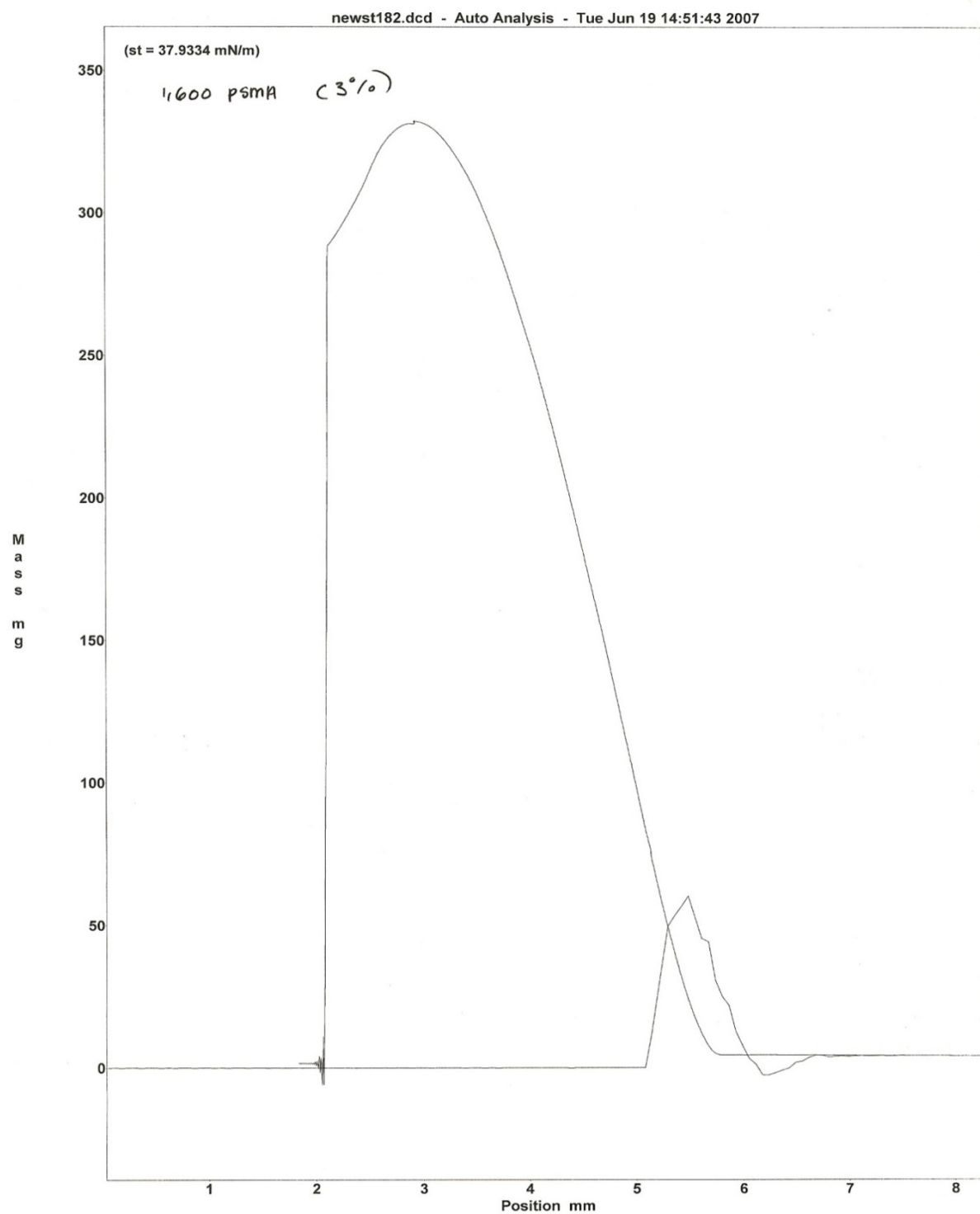


Figure 5.11. The static surface tension (37.9mN/m) of 3% (w/v) PSMA MW 1, 600 - solution.

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Despite having different structures and thus different HLB values (see Table 5.4), all three PSMA-based hydrolysed solutions of varying molecular weights, exhibited a very low static surface tension of  $\leq 42 \text{ mN/m}$ , characteristic of surfactant behaviour. The presence of all three polymer solutions lowered the surface tension of water, at the air-liquid interface, demonstrating that the PSMA-based copolymers show surface activity and adsorption behaviour at the air-liquid interface. The results obtained indicate that the surface activity is dependent on the polymer molecular weight, the maleic acid-to-styrene ratio balance, and is also affected by the presence of additional partial ester functionalities. All these varying factors are known to effect the hydrophilic-lipophilic balance (HLB) and hence the performance of a surface active agent. The PSMA MW 1, 900 partial propyl ester, with a reported maleic anhydride-to-styrene monomer feed ratio of 1: 2, exhibited the lowest static surface tension ( $35.34 \text{ mN/m}$ ) illustrating that the change in the HLB values for the PSMA solutions affects the adsorption from the bulk solution into the air-liquid interface. The greater hydrophobic styrene/partial ester moieties must enhance the adsorption of the PSMA-based solution towards the hydrophobic air and the less hydrophilic maleic acid moieties allows less dissolution into the aqueous bulk media, and hence greater net adsorption. The highest molecular weight PSMA, MW 350, 000 - 10-15% (w/v) mono-partial methyl ester, demonstrates a mean static surface tension that is a little higher ( $41.43 \text{ mN/m}$ ) than the lower molecular weight PSMA-based solutions. This is most likely due to the higher viscosity of the PSMA MW 350, 000 solution, as it is of a much higher molecular weight.

From the rheology analysis (see Figure 5.12), the data obtained shows that the viscosity of the PSMA MW 350, 000 (NR6) is much higher than even the medium molecular weight PSMA MW 120, 000 salt solution. The method employed to measure the static measurement is known as a detachment method, where the du Noüy ring is pulled through the liquid/vapour interface and the maximum downward force is the raw data that is used to calculate the surface tension. This technique fails to take into consideration that highly viscous solutions, i.e., solutions of polymers with very high molecular weight, are in theory going to be more resistant to the du Noüy ring breaking the meniscus, at the liquid-vapour interface. As a consequence, the static surface tension generated would be expected to be higher. One needs to take this into consideration this phenomenon when analysing such data, since no correction factor is employed in this technique for viscous solutions.

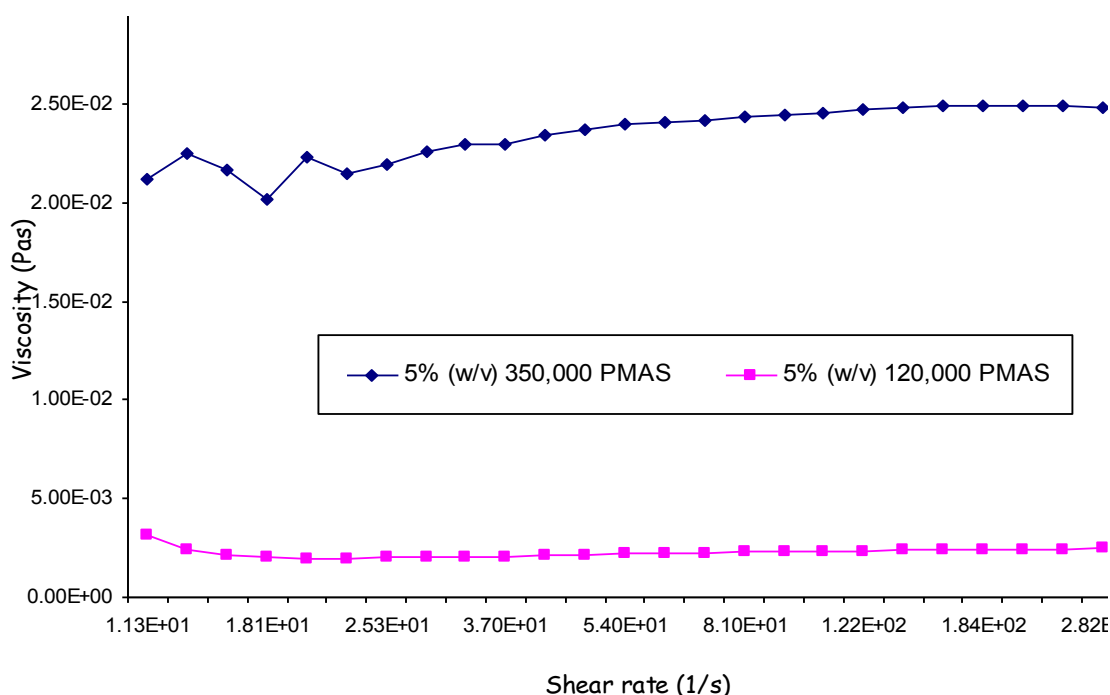


Figure 5.12. The viscosity comparison of 5% (w/v) PSMA MW 350, 000 with the PSMA MW 120, 000.

#### 5.2.4 Langmuir isotherms: measuring dynamic surface tension

The Langmuir-trough from Nima Technology Ltd (Coventry, England, was used to investigate the water soluble Langmuir films of PSMA-based copolymers, at the air-water interface. The Langmuir trough instrument includes a 105cm<sup>2</sup> trough, with two mechanically coupled barriers, a surface pressure sensor, a dipper mechanism (25mm stroke), and an IU4 computer interface with version 5.16 operating software. A 50 microlitre of the hydrolysed PSMA-based solution was injected onto the prior cleaned water subphase, using a Hamilton syringe. Twenty minutes were allowed for solvent evaporation and film equilibration. The film was continuously compressed and then expanded at a constant rate in order obtain isotherm cycles. Isotherm cycles were repeated at least twice to obtain reproducible results. In this study the association behaviour of hydrolysed PSMA-based solution, in aqueous solution was studied onto a HPLC grade subphase surface, at the air-water interface, in a Langmuir trough [90].

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Table 5.7. A summary of the Langmuir isotherm features of some hydrolysed PSMA-based solutions, at room temperature.

<b>3% (w/v) hydrolysed PSMA/ hydrolysis route</b>	<b><math>\pi_c</math></b>	<b>Surface activity</b>	<b><math>L_E</math></b>	<b><math>L_c</math></b>
NR1 (room temp)	14	little	little	no
NR1/>50°C for (48 hours)	24	moderate	little	no
NR8 (room temp)	34	yes	yes	yes
NR8/>50°C (48 hours)	36	yes	yes	yes
NR7/>50°C for (48 hours)	11	hysteresis	little	no
NR7/>50°C (48 hours) (pH 5 buffer solution)	20	yes	little	no
NR3/>50°C(48 hours)	28	yes	little	no
NR8(room temp)	30	yes	no	no

See Chapter 3 (sections 3.8-3.9) for the Aston experimental setup, detailed theory and for further experimental details.

Analysing the Langmuir isotherms obtained for the various hydrolysed PSMA-based solutions revealed some interesting features. Firstly, the method employed for hydrolysis can affect the surface behaviour of the hydrolysed PSMA MW 1, 600 solutions, at the air-water interface. Hydrolysis under alkaline conditions for 48 hours at >50°C, ensures more efficient conversion of the maleic anhydride groups to charged maleic acid groups, in contrast to hydrolysis at room temperature. This seems to be a more important when hydrolysing a PSMA-based solution with a reported styrene-to-maleic anhydride monomer feed ratio of 1:1. This is expected, as there is a higher concentration of maleic anhydride groups to hydrolyse. A greater collapse pressure is attained for the MW 1, 600 PSMA solution (NR1) that was hydrolysed for 48 hours at >50°C. The shape of the Langmuir isotherm was characteristic of a condensed Langmuir isotherm. Figure 5.13 presents the Langmuir isotherm for this PSMA solution; a distinct Liquid condensed phase transition can be seen with a reasonable collapse pressure of 24mN/m (for a surface active agent), exerting a dynamic surface tension of 48.8mN/m, under maximum compression, in the set minimum area. Figure 5.14 exemplifies a typical Langmuir isotherm of PSMA MW 1, 600 hydrolysed, at room temp for 48 hours. The features of this isotherm show a distinct Liquid condensed phase, as well but a lower collapse pressure of 14mN/m. In turn, a lower dynamic surface tension of 58.8mN/m is attained, under maximum compression, in the set minimum volume. Figure 5.13, demonstrates that a higher concentration of charged maleic acid groups leads to an improved hydrophilic and hydrophobic balance. As a consequence, this generates a more effective polyanionic surfactant solution, which

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shows more amphipathic behaviour (adsorption onto the aqueous subphase surface, at the air-water interface). This in turn, leads to greater adsorption and a more stable polymeric film is generated at the air-water interface.

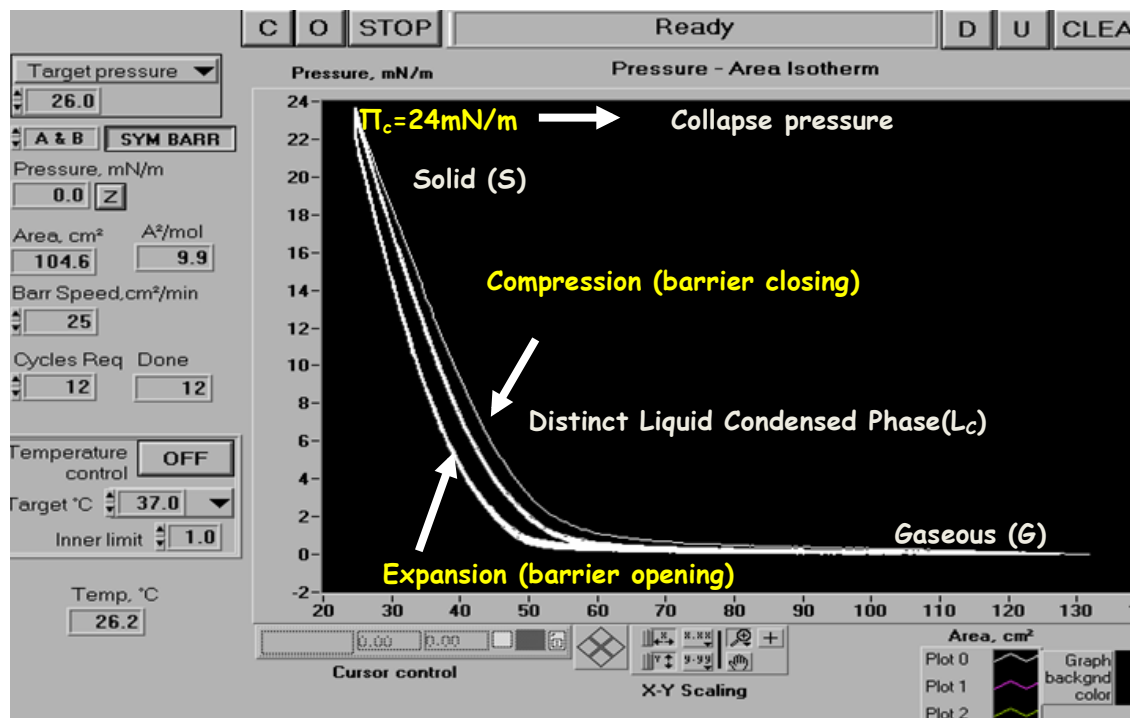


Figure 5.13. A Langmuir isotherm for 50 microlitres of 3% (w/v) hydrolysed PSMA, MW 1,600 (NR1), at 50°C for 48 hours showing a collapse pressure of 24mN/m, a dynamic surface tension of 48mN/m, under maximum compression, in the set minimum volume.

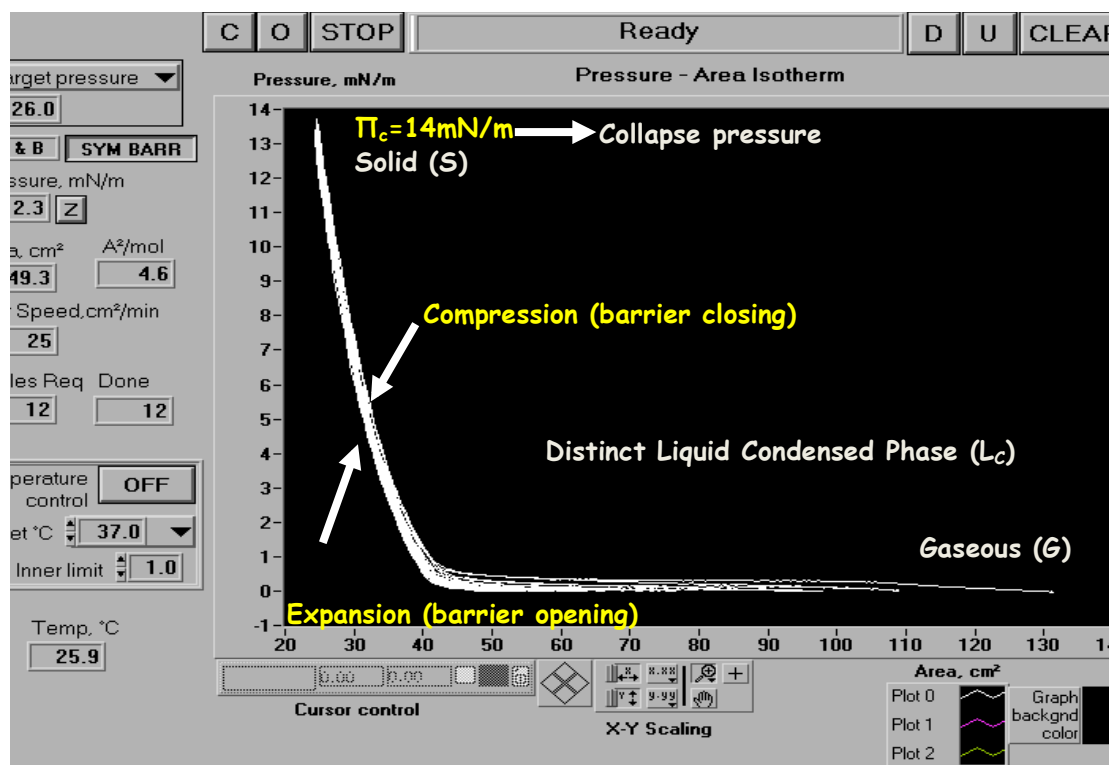


Figure 5.14. A Langmuir isotherm for 50 microlitres of hydrolysed at room temp, PSMA 3% (w/v), MW 1, 600 (NR1), at 25.9°C, with a collapse pressure of 14mN/m exhibiting no hysteresis when compressed and expanded at 25cm²/min.

The very high molecular weight hydrolysed PSMA MW 350, 000 copolymer, available from S-A, exhibits the greatest surface activity; this is portrayed by the fact that the Langmuir isotherms for these polymers solutions show higher collapse pressures, 30mN/m, and 34mN/m and 36mN/m, respectively. This implies that hydrolysed PSMA-based solutions prepared from higher molecular weight PSMA, in particular the PSMA-based copolymer that possesses additional hydrophobic mono-partial methyl ester moieties must thus form more stable polymers films, at the air-water interface. This may be due to a greater radius of gyration, a longer end-to-end distance that generates enhanced surface coverage on an HPLC grade water subphase surface. An extremely fascinating result, definitely worthy of highlighting is observed for the Langmuir isotherm of hydrolysed PSMA, MW 350, 000 - mono-partial methyl ester (10-15% w/v) - NR 6. In this isotherm, there is a distinct Liquid-expanded-to-Liquid condensed ( $L_E$  and  $L_C$ ) transition, (see Figures 5.15) and a rare hysteresis is also present. The only difference in this PSMA copolymer type is the presence of additional mono-partial methyl ester (hydrophobic) moieties that alter the hydrophilic-lipophilic balance. Some of the maleic anhydride functionality (10-15% w/v) for this PSMA-based copolymer, have been partially modified by an esterification reaction, that form mono-partial methyl ester moieties, which results in a more hydrophobic PSMA-based copolymer. Not only, is this PSMA-based copolymer solution easier to hydrolyse, it also forms more effective

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polyanionic surfactants that possess greater amphipathic behaviour. The presence of the hydrophobic additional mono-partial methyl ester functionalities varies the self-assembly of this hydrolysed PSMA-based copolymer solution, orientating the hydrophilic charged maleic acid groups to the hydrophilic water subphase, and the hydrophobic styrene and the additional methyl ester groups towards the hydrophobic air. This hydrolysed PSMA-based solution adsorbs more effectively onto the water subphase surface, at the air-water interface. The partition between the interface and bulk is influenced by the hydrophilic to hydrophobic balance, so less dissolution of the polymer occurs into the bulk as a result of the modified structure. This more hydrophobic macromolecule is an excellent candidate for an effective surface active agent, in particular as a synthetic 'surfactant protein analogue'. This behaviour mimics the functionality of native surfactant proteins that arrange their hydrophilic and hydrophobic groups at opposite facets like an alpha helix coil, as an amphipathic secondary structure.

Figure 5.15, depicts the Langmuir isotherm for this hydrolysed PSMA-based solution, the shape of the isotherm is in fact, and characteristic of a condensed Langmuir isotherm, with an intriguing strong  $L_E$  and  $L_C$  phase transition. A surprisingly high collapse pressure of 34mN/m was attained when it was compressed at 50cm<sup>2</sup>/min (see Figure 5.16) and a collapse pressure of 36mN/m, when it was compressed at a rate of 100cm<sup>2</sup>/min. This polymer solution lowers the surface tension of the clean water subphase surface to a dynamic surface tension of 38.8mN/m and 36.8mN/m, respectively. However, doubling the compression rate for the PSMA film did not largely affect its Langmuir isotherm shape. In fact, this just raised the minimum dynamic surface tension attained, at maximum compression (in the minimum area). This polymer film was fairly resistant to compression. This is an excellent property for a surface active agent, especially for biolubrication applications, as it possesses boundary lubricating capability on a water subphase surface, approaching physiological pH (at the air-water interface). This feature (behaviour) also mimics the functionality of native surfactant proteins. Thus is an example of functional biomimesis being achieved in this MPhil thesis.

Figure 5.16, illustrates the Langmuir isotherm for high molecular weight (MW 350, 000) commercial hydrolysed PSMA salt solution. Although, a condensed isotherm shape is present, a high collapse pressure is obtained (30mN/m), and a minimum dynamic surface tension of 42.8mN/m is still reached, under maximum compression, in the set minimum area. No distinct liquid expanded to liquid condensed phase transition was



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observed. Figure 5.16, discloses that in the absence of the hydrophobic mono-partial methyl ester functionality, the  $L_E$  and  $L_C$  phase transition was absent. This indicates that the partial modification of the maleic anhydride moieties to mono-partial methyl ester functionalities improves the surface activity of the high molecular weight PSMA, by rendering it more hydrophobic and thus modifying its hydrophobic and hydrophilic balance ( $HLB = 17.5$ ), as opposed to the  $HLB$  value of 28, for the unmodified hydrolysed PSMA-based solution.

The presence of the dissolved electrolytes will also retard the electrostatic repulsion between charged maleic acid groups. This will result in favouring of the polymer chains cohering together and hence explain why a more condensed isotherm is formed for the unmodified hydrolysed MW 350, 000 PSMA salt solution. This is an example of how the responsive surface behaviour of hydrolysed PSMA-based copolymers can be modified by partially modifying the maleic anhydride groups. These findings can help us design novel synthetic surfactant protein analogues, specifically for biolubrication applications, which impart boundary lubrication at the air-water interface - characteristic of native surfactant proteins.

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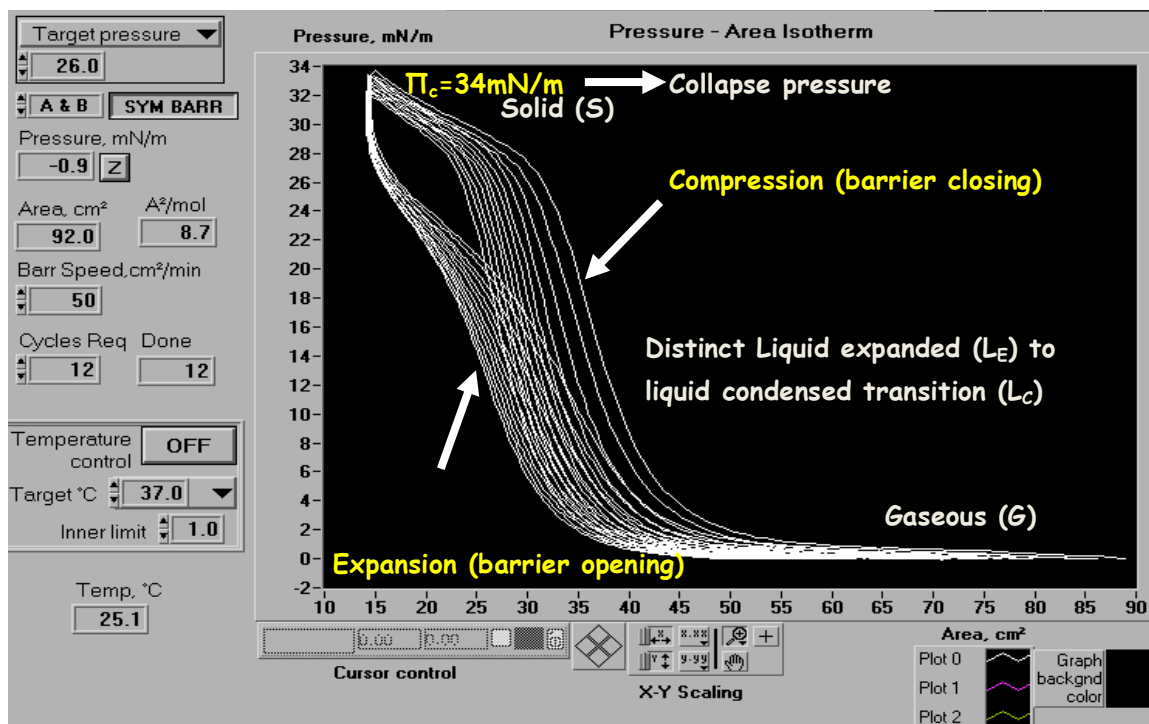


Figure 5.15. A Langmuir isotherm for 50 microlitre of 3% (w/v) of hydrolysed PSMA MW = 350,000 - mono-partial methyl ester (10-15% w/v), at 25 °C, with a collapse pressure of 34mN/m exhibiting hysteresis and a strong  $L_E$  and  $L_C$  phase transition, when compressed and expanded, at a rate of 50cm<sup>2</sup>/min.

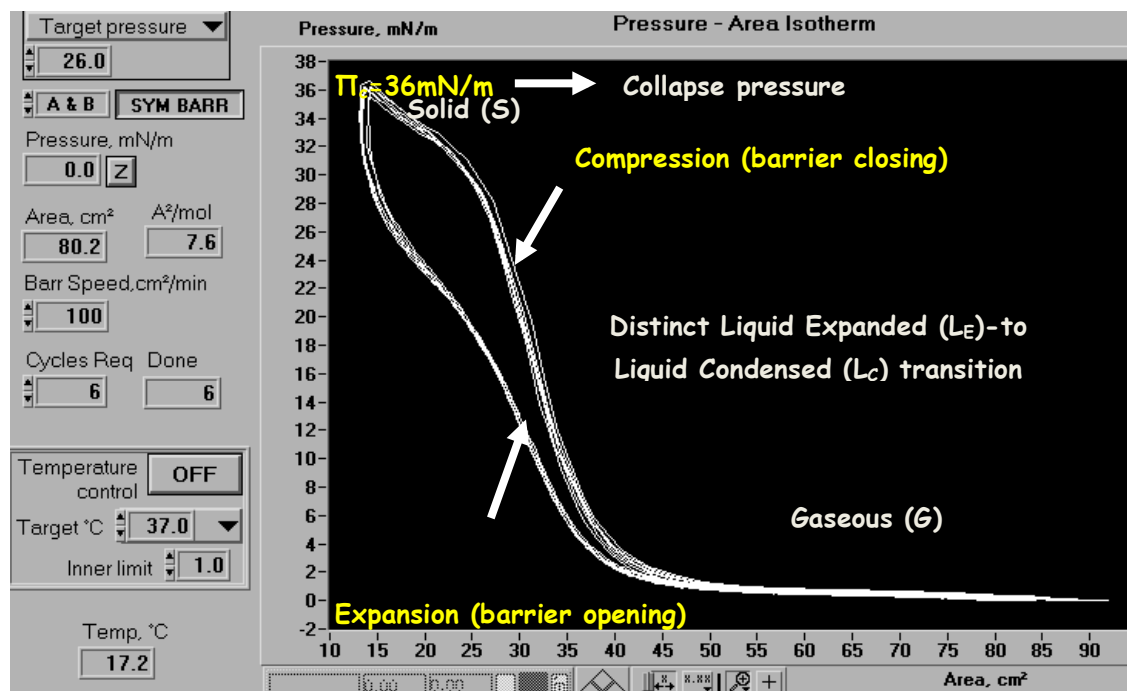


Figure 5.16. A Langmuir isotherm for 50 microlitres of 4% (w/v) hydrolysed PSMA MW = 350,000 - mono-partial methyl ester (10-15% w/v) at 17.2 °C, with a collapse pressure of 36mN/m exhibiting hysteresis at the  $L_E$  and  $L_C$  transition - when compressed and expanded at a rate of 100cm<sup>2</sup>/min.

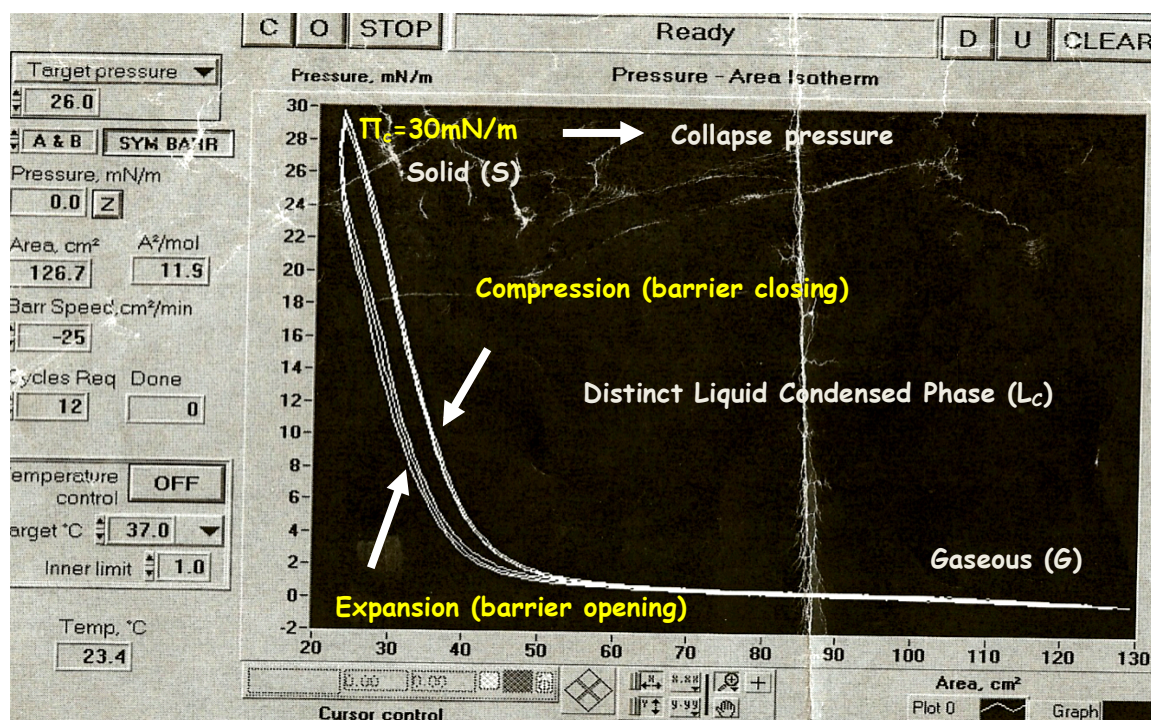


Figure 5.17. A Langmuir isotherm for 50 microlitres of 3% (w/v) hydrolysed PSMA MW = 350, 000 salt solution at 23.4°C, with a collapse pressure of 30mN/m exhibiting a condensed Langmuir isotherm, when compressed and expanded at a rate of 25cm<sup>2</sup>/min.

Do note that slight different conditions have been employed in order to investigate the effects of concentration, the presence of counter ions and the presence of additional functional groups have upon the dynamic surface tension of the PSMA MW 350, 000 based copolymers via Langmuir studies. Interestingly, slight differences can be noted and explained in terms of counter ion effects, concentration and most importantly due to the presence of additional hydrophobic partial ester moieties, altering the shape of the isotherm and hence the collapse pressure attained.

Figure 5.18, illustrates the Langmuir isotherm for hydrolysed PSMA MW 1, 900 - partial propyl ester. A low collapse pressure is obtained (11mN/m) but some rather early hysteresis is visible. The dynamic surface tension of only 61.8mN/m is attained, when the polymer solution is compressed under maximum compression, in a set minimum area. The shape of the isotherm is definitely typical of an expanded isotherm. This may be due to the presence of bulky propyl ester moieties, preventing efficient packing. The hydrophobic propyl ester groups orientate themselves just like the hydrophobic styrene towards the hydrophobic air and the minority (11%) hydrophilic charged groups orientate to the hydrophilic aqueous subphase. This results in the formation of an expanded film. This PSMA is the most hydrophobic (HLB = 6.16) and is not useful in the present context. Nevertheless, it is still important to discuss its surface behaviour,

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at the air-water interface purely for academic interests. The nature of the substrate, particularly with respect to changes in pH, is important when the monolayer is ionisable. When spread on alkaline substrates, because of the ionisation and consequent repulsion between the carboxyl groups, films form gaseous and or liquid expanded films at much lower temperatures. Dissolved electrolytes in the substrate can also have a profound effect on the state of the film. In acidic conditions, at pH 5 the PSMA MW 1, 900 - partial propyl ester (NR 9) seems to come out of solution and adsorb onto the surface, exhibiting greater surface activity and adsorption capability. This is because the charged maleic acid groups become less ionised and therefore are less soluble in the acidic subphase. The level of hydrogen bonding is enhanced through intramolecular and intermolecular hydrogen bonding. A hypercoiling phenomenon is likely to be apparent, having a greater tendency to adsorb at the aqueous subphase surface. Additionally, hydrophobic moieties would still orientate themselves to the hydrophobic air, so a better hydrophobic and hydrophilic balance is achieved. Hence, a greater collapse pressure of 20mN/m is obtained, and a lower dynamic surface tension of 52.8mN/m is achieved, under maximum compression in the set minimum area. Moreover, a more expanded film is generated, as can be seen in Figure 5.19. Hysteresis in a Langmuir isotherm, is due to resistant of a film to expression, at the air-water interface under repetitive mechanical compression. Hysteresis is also apparent in the isotherm illustrated in Figure 5.18. This is caused by a film resisting compression at the air-water interface, in turn demonstrating surface activity behaviour.

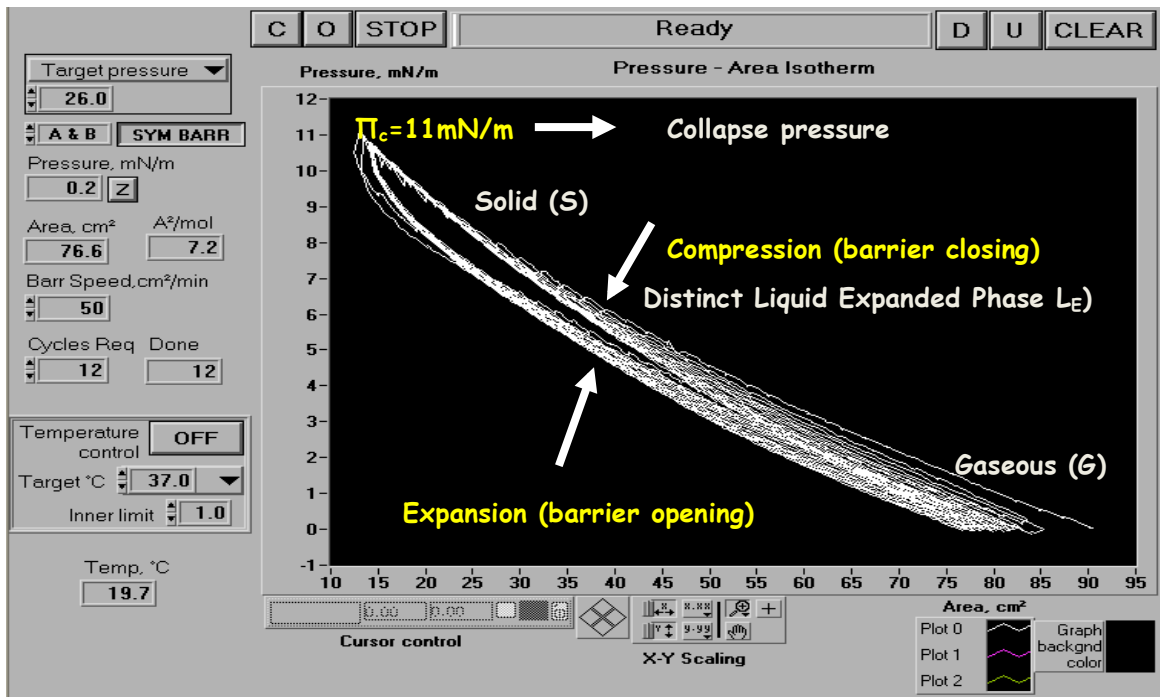


Figure 5.18. A Langmuir isotherm for 50 microlitres of 3 % (w/v) of PSMA MW 1, 900 - partial propyl ester, at 19.7°C, with a collapse pressure of 11mN/m, exhibiting hysteresis when compressed and expanded at 50cm<sup>2</sup>/min.

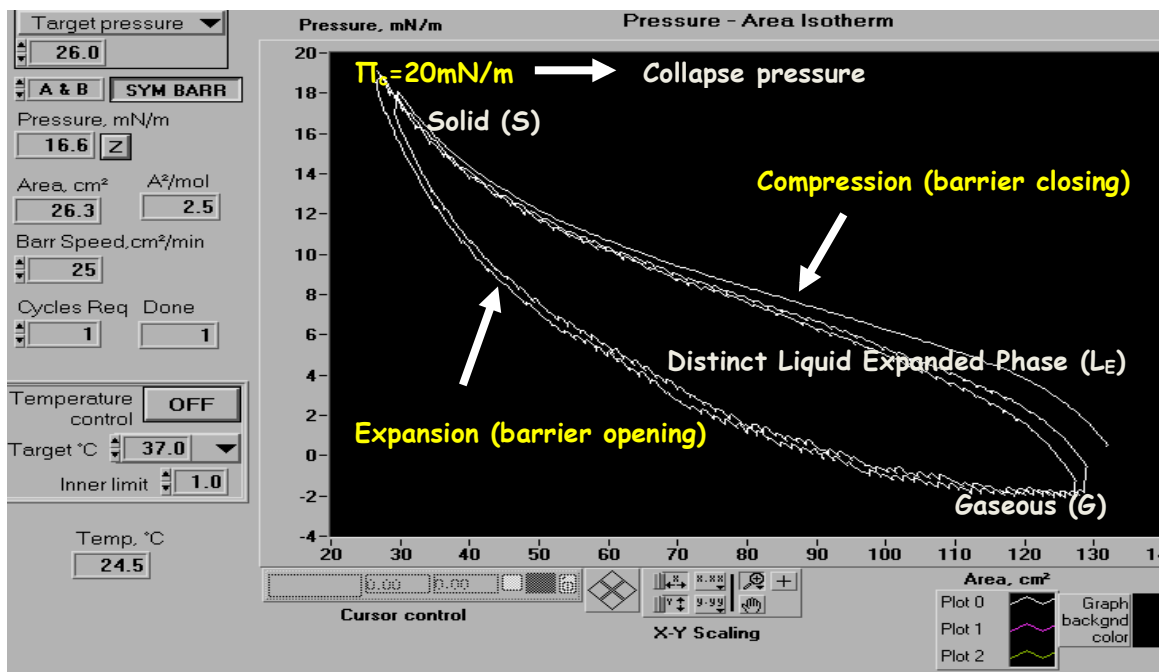


Figure 5.19. A Langmuir isotherm for 50 microliters of hydrolysed 3% (w/v) PSMA MW 1,900 - partial propyl ester, at 24.5°C, with a collapse pressure of 20mN/m, exhibiting hysteresis when compressed and expanded at 25cm<sup>2</sup>/min, at pH=5 (pH=5, phosphate buffer as aqueous subphase).

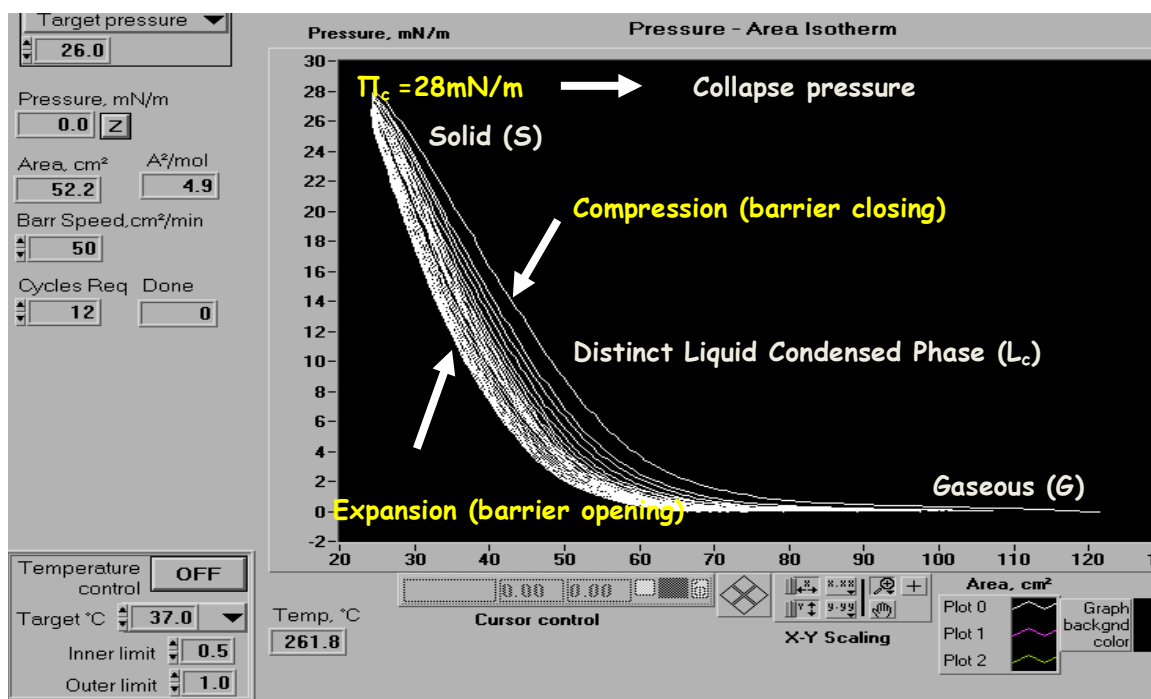


Figure 5.20. A Langmuir isotherm for 50 microlitres of 3% (w/v) of blocky hydrolysed PSMA MW 9, 500 (3:1), at room temp, with a collapse pressure of 28mN/m, exhibiting little hysteresis when compressed and expanded, at a rate of 50cm<sup>2</sup>/min.

Examination of the Langmuir isotherm, under maximum compression, in the set minimum area, for the lower molecular weight PSMA MW 9, 500 (styrene-to-maleic anhydride, monomer feed ratio of 3:1) purchased from POL, shows a little more surface activity (see Figure 5.20) than the so called 'alternating' PSMA MW 1, 600, styrene-to-maleic anhydride monomer feed ratio of 1:1, NR1 (see Figure 5.13). The one difference in the two PSMA-based copolymers is that the former is of a higher molecular weight than the latter. The other difference is that they have dissimilar estimated HLB values; 28 and 18.48, respectively, due to the varying styrene-to-maleic anhydride ratios. For the more hydrophobic PSMA (see Figure 5.20) a greater collapse pressure of 28mN/m is obtained and more hysteresis is observed, demonstrating enhanced adsorption and greater surface activity at the air-water interface. In turn, a dynamic surface tension of 44.8mN is achieved under maximum compression, in the set minimum area.

Table 5.8, summaries the surface behaviour of some interesting hydrolysed PSMA-based solutions studied (via the Langmuir trough) at the air-water interface, with varying estimated HLB values. Ultimately, what can be deduced from the data collected is that the surface behaviour of hydrolysed PSMA-based solutions (at the air-water interface) can be predicted from the PSMA structure. This is informative when designing hydrolysed PSMA-based copolymers, for specific applications. The



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estimated HLB values correlate well with the numerical surface measurements obtained. The lipid solubilising agent prepared from the highest molecular weight PSMA MW 350, 000 - with additional hydrophobic, mono-partial methyl ester functionalities (10-15 % w/v), showed the most effective surface activity, at the air-water interface. This agrees well with its estimated HLB value (17.5). The Langmuir isotherm shape was intriguing and demonstrated superior characteristics (see Figures 5.15-5.17 for detailed explanation of the analysis of the Langmuir isotherms obtained). Furthermore, by preparing hydrolysed PSMA-based copolymers under more vigorous conditions (at > 50°C), for a longer duration (at least 48 hours) optimises surface properties. What was also established was that, the surface behaviour of the hydrolysed PSMA-based solutions is pH dependent. In more acidic conditions the hydrolysed PSMA, as expected, comes out of solution and adsorbs at the aqueous water subphase surface. It must also be noted that the novel lipid solubilising agents (hydrolysed PSMA-based copolymers) are responsive to changes in pH. Additionally, in the presence of dissolved electrolytes, more condensed isotherms are formed. This implies that in the presence of counter ions (e.g., sodium, potassium or calcium free in solution), the electrostatic repulsion of the charged maleic acid groups is retarded, due to the shielding effects of the dissolved counter ions.

Table 5.8. A summary of the PSMA Langmuir isotherm features (ST=surface tension)

<b>3%(w/v) hydrolysed PSMA</b>	<b><math>\pi</math> (dynamic ST) / <u>static ST</u></b>	<b>Surface activity</b>	<b>HLB</b>
NR1 (room temp)	14 (58) / <u>37.8</u>	little	28
NR1 (>50°C for 48 hours)	24 (48)	moderate	28
NR 6 (room temperature)	34 (38.8) <u>41.3</u>	yes	17.5
NR6 (>50°C /48 hours)	36(36.8)	yes	17.5
NR7 (>50°C /48 hours)	11(61.8)	hysteresis	6
NR 7(>50°C (48 hours)/ pH 5 buffer solution	20(52.8)/ <u>35.3</u>	yes	6
NR 3 (>50°C /48 hours)	28(44.8)	yes	18.4
NR 8 salt solution	30(42.8)	yes	<28

### 5.2.5 Frictional behaviour: measuring coefficient of friction

A 100 microlitres quantity of the selected hydrolysed PSMA-based solution was placed onto the CSM Nano Scratch Bio-tribometer table using a micropipette. See Chapter 3 (section 3.4), for experimental set up and further experimental details. The frictional

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behaviour of the two hydrolysed PSMA-based solutions investigated is described below:

- A) 10% (w/v) of PSMA MW 120, 000 sodium salt solution,
- B) 10% (w/v) of PSMA MW 350, 000 - 10-15% mono-partial methyl ester.

These two samples were selected as they exhibited desirable surface properties, as already discussed earlier in this chapter. The frictional force of the sliding polypropylene (PP) head against the solution (hydrolysed PSMA-based solution, under investigation) placed onto the Melinex sheet (polyethylene terephthalate) was recorded as a function of distance travelled in (mm 0-20mm). A load of 60mN (normal force) at a speed of 30mm/min was used. The only variable that was changed was the lubricant (hydrolysed PSMA-based solution). HPLC grade water was also tested as the control for the experiments. For each test the run was repeated 10 times, and each test was repeated at least three times, with fresh lubricant and Melinex sheet in order to obtain concordant results. The overall mean coefficient of friction, the mean static and the mean dynamic coefficient of friction was calculated. The mean static coefficient was taken from the 0-4mm, and the mean dynamic coefficient of friction from 8mm-12mm, and the overall mean coefficient of friction from 0-20mm, respectively. The standard deviation was also calculated to review the viability of the data obtained. All values are summarised below in Table 5.9. The standard deviation values obtained are low indicating reliable data was obtained.

Table 5.9. A summary of the frictional data obtained with PP and Melinex. It summaries the mean coefficient of friction, static, dynamic and static/dynamic coefficient of friction, respectively. The standard deviation (SD) of the data is also stated.

<b><i>Lubricant</i></b>	<b><i>Mean</i></b>	<b><i>Static</i></b>	<b><i>Dynamic</i></b>	<b><i>Static/Dynamic</i></b>	<b><i>SD</i></b>
HPLC water	0.48	0.5	0.47	1.08	0.01
NR 6-MW 350, 000	0.22	0.2	0.26	1.27	0.05
NR 5-MW 120, 000	0.56	0.56	0.57	0.97	0.01



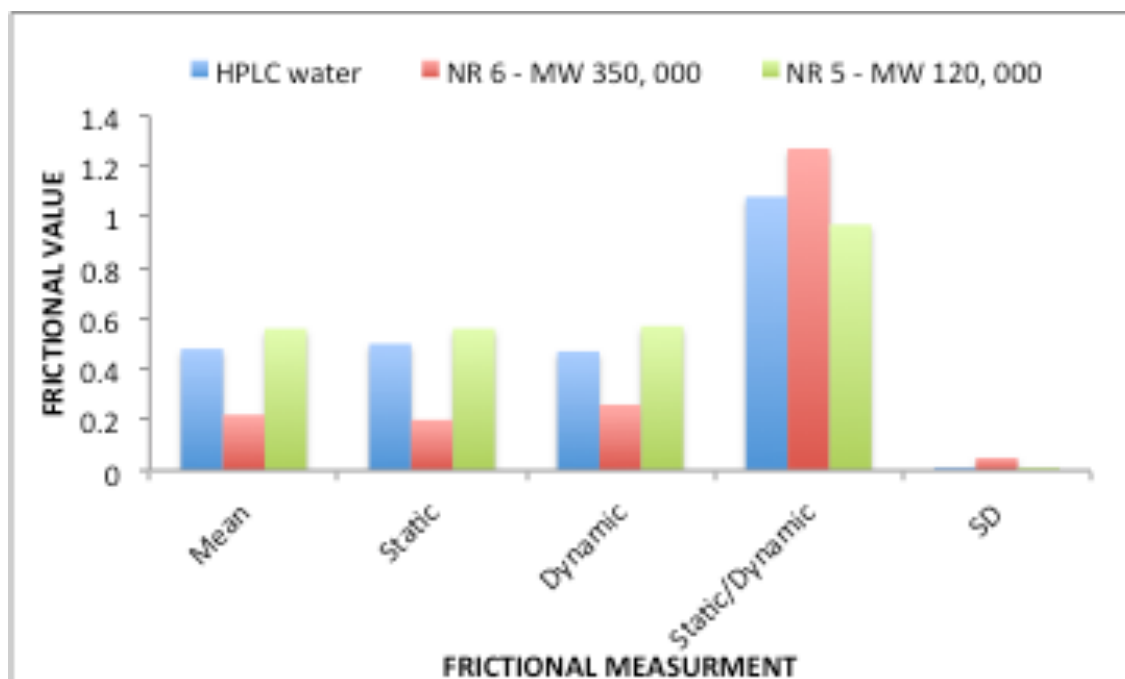


Figure 5.21. A summary of the frictional behaviour for hydrolysed PSMA-based solutions on PP and Melinex sheet; mean coefficient of friction, static, dynamic and static/dynamic respectively. The standard deviation (SD) of the data is also stated.

From the data obtained, the mean coefficient of PSMA MW 350,000 - mono-partial methyl ester (NR6) is a lot lower (0.22) than the medium molecular weight PSMA MW 120,000 salt solution (0.56). There does not seem to be much difference in the static or dynamic coefficient of friction for both lubricant systems. One does not expect the coefficient to be low, as the frictional force test is performed on a hydrophobic PP head surface. There are two factors that can potentially explain the differences in the frictional behaviour of the two different lubricants observed. Firstly, the molecular weight of the PSMA and secondly the surface activity which is related to the chemistry (the chemical structure) of the hydrolysed PSMA-based solution.

Figure 5.23, illustrates that the viscosity of the two PSMA-based solutions vary, the higher molecular weight PSMA having a higher viscosity at 37°C. However, both solutions are viscoelastic. The hydrolysed PSMA solutions exhibit Newtonian rheological behaviour, i.e., the viscosity is independent of the shear rate. Hydrolysed PSMA MW 350,000 - mono-partial methyl ester, not only demonstrates ease of hydrolysis, i.e., excellent solubility in water; it is additionally viscoelastic and amphipathic. These features are essential for an effective biological surfactant or boundary lubricant. It has the ability to act both as a hydrodynamic lubricant (as it is viscoelastic) and a boundary lubricant (surface active) and therefore can perform as an effective biological lubricant/surfactant protein analogue. As it is surface active, evidence from which is supported by its Langmuir isotherm, see Figure 5.15 (a collapse

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pressure of 34mN/m, and a strong  $L_E$  and  $L_C$  transitions present). It has the capability to adsorb onto the hydrophobic PP surface (orientation of the hydrophobic styrene and ester moieties occur towards the hydrophobic PP) and therefore provide boundary lubrication. In order to achieve lower coefficients of friction, a lubricant must operate in the both hydrodynamic and the boundary lubrication regime (refer to the Stribeck curve explained earlier, in Chapter 1). Both mechanisms need to occur simultaneously, in competition and support each other in order to operate in the mixed lubrication regime, and therefore attain low coefficients of friction. The investigation of the effects of substrate was also conducted, a hydrophilic, a high water content contact lens (Focus Dailies™) was placed onto the PP head. The Focus Dailies™ contact lens (-3.0 D) had been dehydrated for 5 minutes after being removed from the packing solution. The frictional test was repeated, as described previously, under the same conditions. Figure 5.24, shows that the medium molecular weight hydrolysed PSMA MW 120, 000 salt solution behaves differently on a hydrophilic surface. The overall mean coefficient of friction is far lower  $<0.1$ , as opposed to a hydrophobic surface (0.56). This demonstrates that the surface properties of a lubricant have a large impact on the frictional behaviour at surfaces, at the liquid-solid interface. The rich hydrophilic moieties produced by the charged maleic acid in the hydrolysed PSMA-based polymer provide strong attraction to the hydrophilic, neutral Focus Dailies™ contact lens surface. This experimental observation and findings suggest that these hydrolysed PSMA-based solutions may prove to be effective lubricants, on such smooth, flexible and hydrophilic neutral surfaces and substrates.

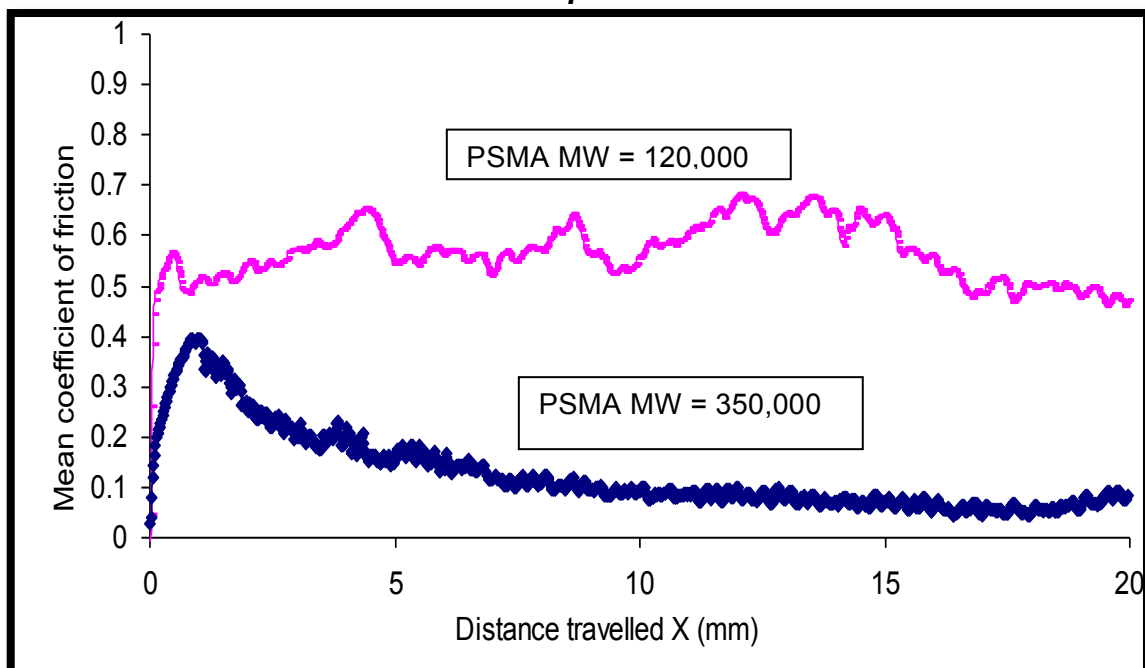


Figure 5.22. The mean coefficient of hydrolysed PSMA solutions (PSMA MW = 120, 000 salt solution (pink/higher trace), PSMA = MW 350, 000 - mono-partial methyl ester (10-15% w/v) (blue/lower trace), against distance travelled.

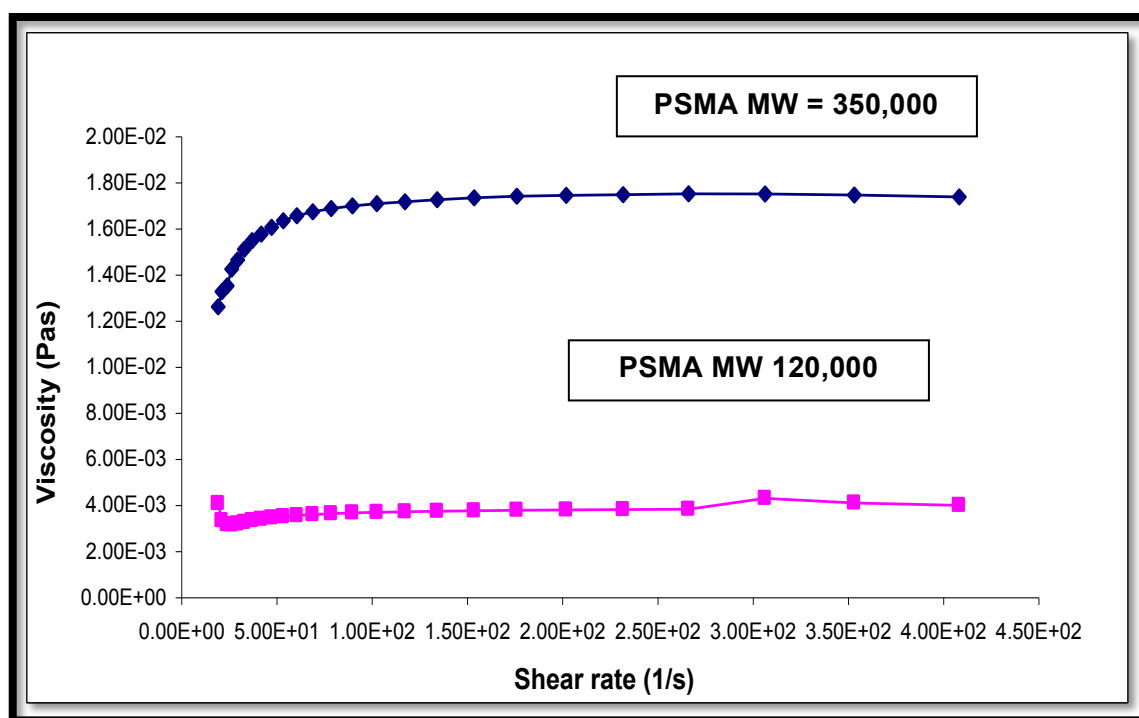


Figure 5.23. The viscosity of 10% (w/v) of hydrolysed PSMA-based solutions, against shear rate, at 37°C.

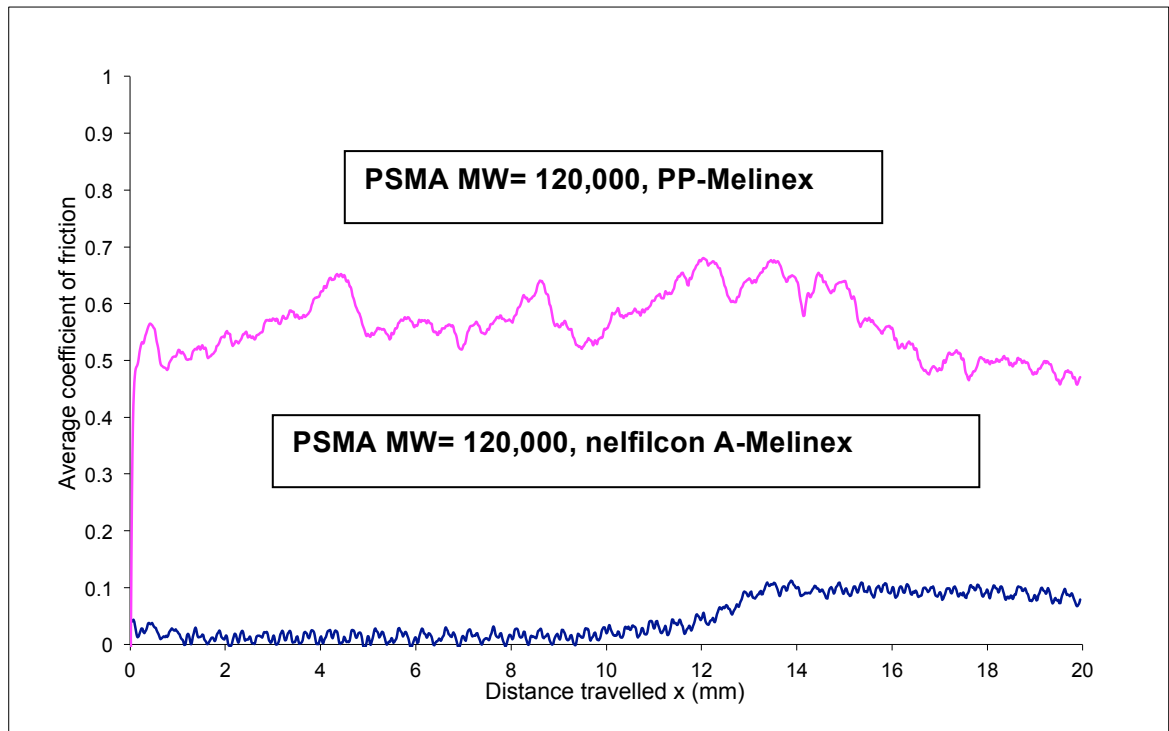


Figure 5.24. The frictional behaviour of hydrolysed PSMA MW = 120,000 salt solution, with a hydrophilic Dailies contact lens (nelfilcon A - blue), and a hydrophobic PP (Pink) surface.

### **5.3 Discussion**

Novel lipid solubilising agents (polyanionic surfactants) have successfully been synthesised from a broad range of PSMA-based copolymers (see Table 5.4 for all lipid solubilising agent synthesised in the study, with respected estimated HLB values. The ease of hydration and hydrolysis for the PSMA-based copolymer was structure and more importantly, microstructure dependent. From experimental observations, it seems that molecular weight has little influence on the ease of hydrolysis during the formation of the novel lipid solubilising agents synthesised in this study. The presence of methyl ester groups (modified maleic anhydride moiety) enhanced the rate and ease of the hydrolysis and hydration; which can be explained in terms of hydrogen bonding effects. The presence of hydrophobic, mono-partial ester groups effects the strength and nature of hydrogen bonding, in that it changes the balance of intermolecular and intramolecular hydrogen bonding, which in turn affects the critical pH of the lipid solubilising agent.

The ultimate aim of this study was to design polyanionic surfactants that possess a critical pH, approaching physiological pH. Essentially, this is governed by a balance of electrostatic attraction and by the hydrophobic effect. This was achieved by hydrolysing PSMA-based with higher styrene content and more importantly with those materials that exhibited additional (hydrophobic) mono-partial ester moieties. Although, the presence of higher styrene content slowed the rate of hydrolysis, the higher styrene content is desirable, as more hydrophobic synthetic surfactant proteins analogues are generated from these materials, that possess higher critical pH values, and a greater hydrophobic and hydrophilic balance is also achieved. More effective surfactant protein analogues are thus synthesised from these PSMA-based copolymers, evidence of this has been demonstrated from the surface chemistry analysis of these material reported in this thesis which correlates well with the estimated HLB values. The presence of higher styrene content and more importantly, the presence of hydrophobic additional mono-partial ester moieties change the hydrophobic-hydrophilic balance, as expected.

Synthetic surfactant protein analogues have been successfully prepared with the desired estimated HLB values, as effective surface active agents. These are ideal candidates for biological surfactants, in that they not only act as lipid solubilising agents, but also function as boundary lubricating agents too.

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The data generated in Table 5.4 indicates that a broad range of PSMA-based copolymers, are now available, which extends their applications range. The information generated is a good starting point that can be utilised to predict the behaviour of hydrolysed PSMA-based copolymers and thus is useful when selecting and designing PSMA-based copolymers, for specific application requirements.

All hydrolysed PSMA-based copolymers exhibit very low static surface tension values measured via the Pt-Ir, du Noüy ring method. However, the dynamic surface tension values obtained via Langmuir techniques seemed to correlate well with the theoretical HLB values estimated. Furthermore, the method of hydrolysis also affected the surface properties of the resulting lipid solubilising agent. The presence of mono-partial ester moieties has the greatest effect on the performance of a synthetic surfactant protein analogue; for example, the dynamic surface tension at the air-aqueous interface, as measured using the Langmuir technique.

In this work, the strategic approach undertaken for designing and synthesising novel lipid solubilising agents has been achieved by using functional biomimesis. Novel hydrophilic and hydrophobic synthetic surfactant protein analogues have successfully been developed with desired properties capable of solubilising polar phospholipids, at higher critical pH values, that approach the target physiological pH and which also demonstrate boundary lubrication capability too.

Figure 5.25 shows, the expected molecular orientation for the hydrophilic (maleic acid) and the hydrophobic (styrene and partial ester) at the air-water interface.. This shows how the behaviour of the various types of PSMA-based copolymer segments is dependent on the monomer sequence (triad sequence).

A) For the 1:1 molar ratio PSMA-based copolymer - NR1 (according to the NMR experiments conducted in Chapter 4) may have alternating triad regions amongst the PSMA polymer chains. For this segment the hydrophobic styrene groups will orientate towards the hydrophobic air and the hydrophilic maleic acid will be exposed into the hydrophilic water subphase.

B) For the SEMI-ALT region, more hydrophobic styrene functional groups will be exposed to the hydrophobic air, in turn more hydrophobic regions will be available for hydrophobic interactions amongst various chains adopting the 3D conformation of the polymer chains, at the air-water interface.

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C) Furthermore, for the NON-ALT regions, longer hydrophobic styrene segments will be available at the air-water interface for stronger hydrophobic interactions.

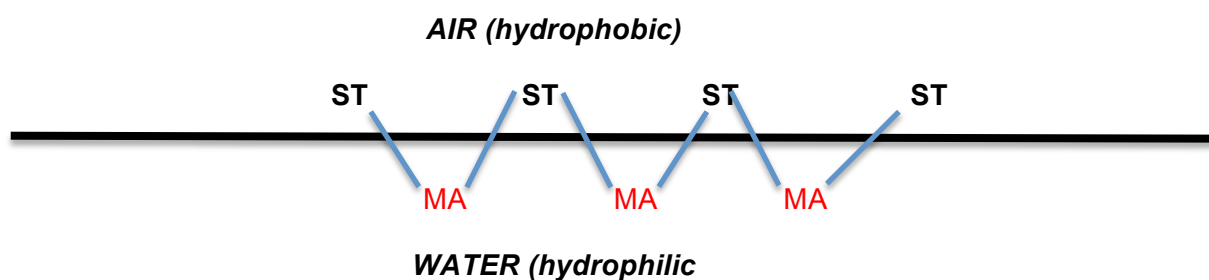
D) Last but not least, the SEMI-ALT (partial ester) region, which will only be present in partial ester PSMA-based copolymers (evidence supported by the NMR data generated in Chapter 4) will possess additional hydrophobic partial ester tails that will orientate also towards the hydrophobic air.

Overall, what is apparent is that the broad range of phospholipid solubilising agents that have been developed in this thesis, which will interact with the polar phospholipids (DLPC/DPPC) uniquely. The hydrophobic materials will have more regions for interaction with the hydrophobic DLPC/DPPC tails, and more so the partial ester materials that possess additional hydrophobic ester tails will provide another area of contact for the hydrophobic polar phospholipid tails, in order to render them aqueous soluble, at specific pH. Additionally, the higher styrene content materials, which will have more NON-ALT PSMA-based segments (as shown in example C in Figure 5.25) and hence a larger hydrophobic styrene area for the hydrophobic phospholipids tails to interact with. All these factors will have consequences on the self assembly and the nature of PSMA-phospholipid complexes that are formulated, with the respected phospholipid solubilising agent.

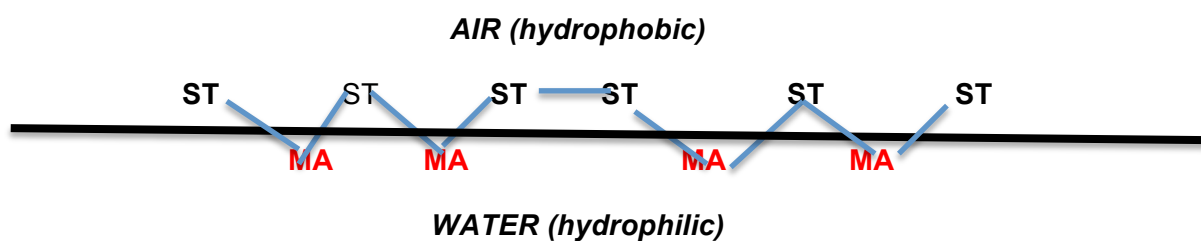
Upcoming Chapter 6 is concerned with development of novel polymer phospholipid complexes, from the various lipid solubilising agents synthesised in this study with and without hyaluronic acid (a natural GAG).

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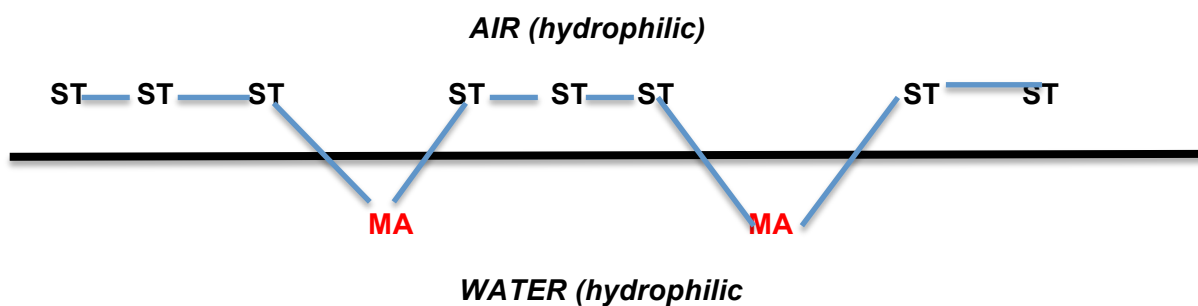
### (A) ALT – PSMA REGION, AT AIR-WATER INTERFACE



### (B) SEMI-ALT PSMA REGION AT AIR-WATER INTERFACE



### (C) NON-ALT PSMA REGION, AT AIR-WATER INTERFACE



### (D) SEMI-ALT (partial ester), PSMA REGION AT AIR-WATER INTERFACE

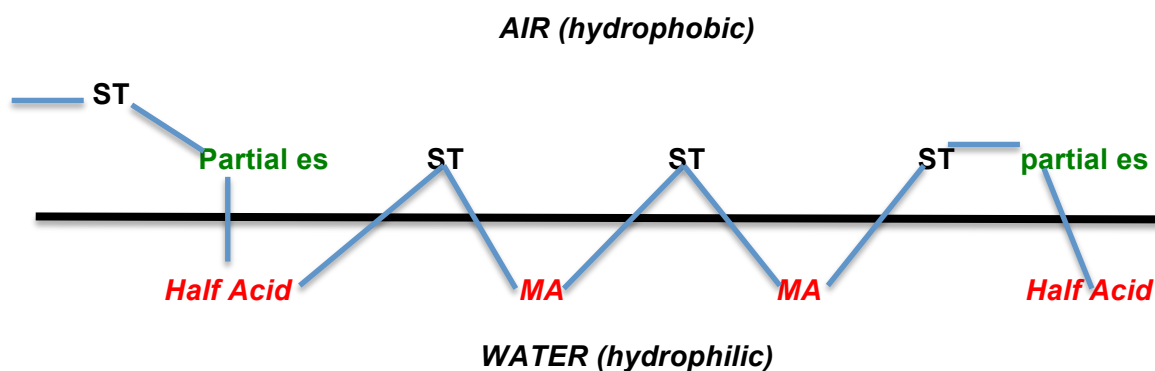


Figure 5.25. Molecular orientation of maleic acid, styrene, partial ester functional groups, at the air-water interface, for (A) ALT (B) SEMI-ALT (C) NON-ALT and (D) SEMI-ALT (partial ester) PSMA-based copolymers



**CHAPTER 6:  
BIOSURFACTANTS; NOVEL  
SYNTHETIC PROTEIN-PHOSPHOLIPID  
COMPLEXES**

## **6.0 Introduction**

This chapter addresses the main aim of this thesis, which has been to design and develop novel biosurfactants (a biosurface treatment) for the management of lubricant deficient diseases, as already outlined in Chapter 1. The employment of HA in orthopaedic and ophthalmic biolubrication applications is evaluated in Chapter 7. The challenge has been to combine HA with boundary lubricant agents. A broad range of lipid solubilising agents (hydrolysed PSMA-based solutions) have been successfully sought and prepared - see Chapter 4 and 5 for full details. The *in-vitro* surface properties for these materials have been measured and assessed for boundary biolubrication and Lipoidal solubilisation applications.

Section 6.1, analyses the surface properties of polar phospholipids via Langmuir and BAM techniques. Section 6.2, then discusses other commercial attempts for Lipoidal supplementation and assesses the surface properties of these products. The surface properties of a native ocular Lipoidal film (extracted from a worn balafilcon A lens), is also studied (for the first time), using Langmuir/BAM. Section 6.3, describes the Aston approach for Lipoidal supplementation. Section 6.4, defines the methodology and explains the novel chemistry for the Astosomes technology. The materials synthesised have been successfully freeze dried and the potential for commercialisation thus warrants further investigation.

Furthermore, the advantages of incorporating HA into the biosurfactants developed are also discussed. This is strongly supported with contemporary reported literature from HA researchers. Finally, some interesting PSMA-DLPC/DPPC based complexes, (with and without HA) are characterised in sections 6.4-6.6. The *in-vitro* surface, rheological and frictional properties of the novel biosurfactants developed is discussed. Although, promising research findings are reported, further investigation is necessary for optimising the biosurfactant formulations developed and for exploring the potential of commercialisation.

Many and possibly all of the surfaces found in biological systems are hydrophilic and fully wettable, and some appear to share common molecular lubricants characterised by phospholipid-apoprotein assemblies (Lipoidal films). The lack of these assemblies results in lubrication deficient states or diseases. The purpose of these materials is to both wet and provide boundary lubrication, while protecting sensitive

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underlying epithelial tissues from damaging shear forces and focal adhesion. However, there are similarities and contrasting differences between the body sites, where such lipid-protein assemblies (Lipoidal films) have been identified. The tear film coats the ocular surface; the pulmonary surfactant covers the alveoli surface in the lungs, and the synovial fluid lubricates the articular cartilage. These fluids exhibit remarkable properties; therefore synthetic mimics to treat lubricant deficient diseases should have comparable properties in order to fulfil a similar function. Thus, the ultimate approach has been functional biomimesis. Bodily lubricants have many similar functions and are the outer fluid layer at the surfaces, preventing focal adhesion from occurring and minimising the transmittance of shear forces to delicate underlying tissue. These fluids exhibit remarkable similarities in their composition, suggesting nature has formulated a similar recipe. All the biological fluids contain lipids, especially phospholipids in combination with proteins. The polar phospholipids may act as the universal lining agent over all internal surfaces of the body although they are too hydrophobic to spread spontaneously from aqueous solutions and form lamellar sheets at interfaces. An amphipathic apoprotein is required to organise the lipid into a bipolar lamellar form for adsorption at interfaces.

We have sought (at Aston) copolymers that mimic the function of native apoproteins by using readily available synthetic polymers to solubilise lipid bilayer in sheets or lamellar form. The aim is to apply the artificial lipid-polymer combinations as a completely new treatment modality. This technology offers a new treatment therapy for lubrication deficient diseases or states: such as dry eye, respiratory distress syndrome and degenerative joint diseases - osteoarthritis (OA) and coatings for prosthetic joints. This chapter describes new synthetic lipid-polymer assemblies and explores the potential applications of this technology to biomaterials. The use of lipids for lubrication presents a problem in aqueous biological environments. Lipids are effectively insoluble in water. Biological systems have overcome this problem by adopting a single approach, which evolved at a primordial stage. Lipids are transported and spread within the body by apoproteins. Apoproteins possess a secondary structural motif of an alpha helix coil, where the amino acid chains are arranged with hydrophobic groups exposed on one facet, perpendicular to the axis of the helix, and hydrophilic groups arranged at the opposite facet, i.e., an amphipathic structure [44-45]. Chapter 4 & 5 has already sought and developed novel lipid solubilising agents, which show desirable characteristics.

## 6.1 Langmuir isotherms of polar phospholipids.

DLPC and DPPC were purchased from Genzyme Pharmaceuticals (now Corden Pharma Switzerland LLC) and were utilised without further purification (see chapter 2, Figure 2.6, for the chemical structures of the polar phospholipids utilised in this study. The surface properties of the polar phospholipids were assessed via Langmuir trough techniques. The polar phospholipids were dissolved into 99% pure chloroform ( $\text{CHCl}_3$ ). A 1% DLPC/DLPC solution was made up (1% w/v) in  $\text{CHCl}_3$  and was spread onto a clean HPLC grade water subphase surface, using a Hamilton syringe (50 microlitres), in a dropwise manner. The spreading solvent, ( $\text{CHCl}_3$  in this case) was allowed to evaporate in order to generate a homogenous DLPC/DPPC film. The film was then compressed and expanded repetitively (at  $22.3^\circ\text{C}$ ) in order to record the Langmuir isotherm of the DLPC/DPPC films. In the trough, biolubricants are exposed to an *in-vitro* ocular surface 'like' interface (air-aqueous). If amphipathic, the hydrophobic moieties orientate towards the hydrophobic air and the hydrophilic heads towards the aqueous hydrophilic bulk subphase (HPLC grade water).

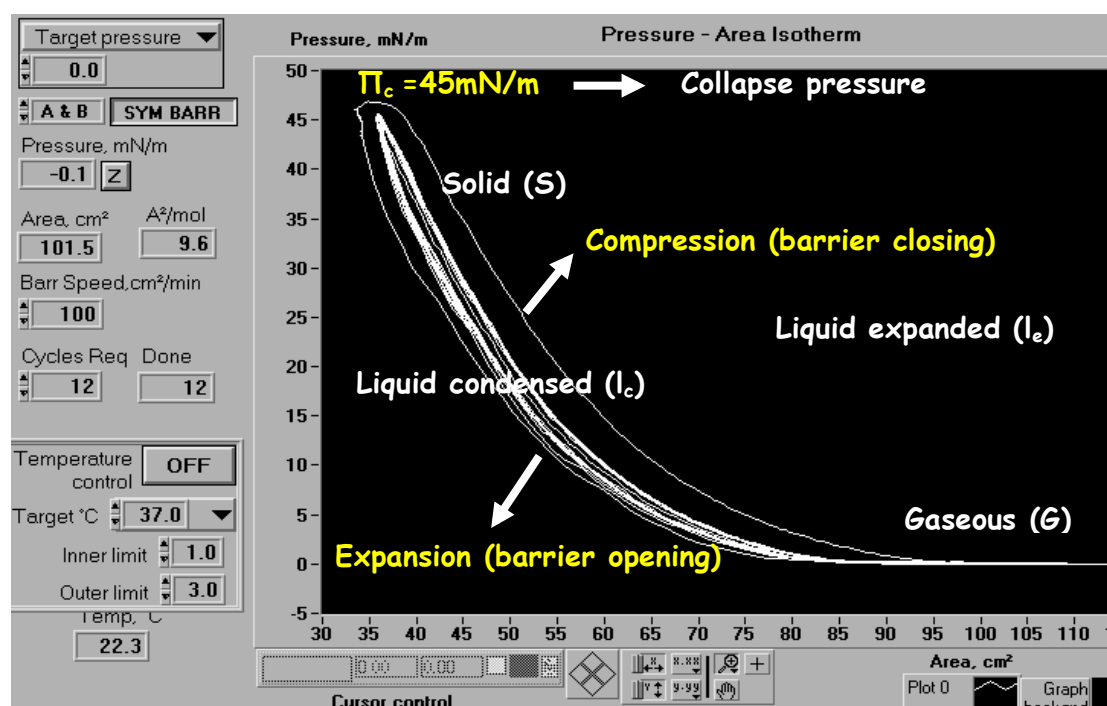


Figure 6.1. A Langmuir isotherm of DLPC in 1% (w/v)  $\text{CHCl}_3$  at  $22.3^\circ\text{C}$ , exhibiting surface activity, and a high collapse pressure of  $45\text{mN/m}$  at the air-water interface, when compressed at a rate of  $100\text{cm}^2/\text{min}$ .

Figure 6.1 shows a Langmuir isotherm of 1% (w/v) DLPC deposited from chloroform onto an aqueous sub-phase, exhibiting amphipathic behaviour, with a collapse pressure as high as  $45\text{mN/m}$  (a dynamic surface tension of  $27\text{mN/m}$ , under maximum compression in the set minimum area) at the air-water interface. In this

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film layer the hydrophobic tails orientate themselves to the hydrophobic air and the hydrophilic heads are towards the hydrophilic water subphase surface. Figure 6.2 portrays a Langmuir isotherm of 1% (w/v) DPPC, exhibiting amphipathic behaviour, with a collapse pressure of 55mN/m at the air-water interface; again the hydrophobic tails orientate themselves to the hydrophobic air and the hydrophilic heads towards the hydrophilic water subphase (in a Langmuir trough). The Langmuir isotherms of the two polar phospholipids demonstrate that they are both surface active at the air-water interface. Nonetheless, it must be noted that this is when they are spread (delivered) with chloroform (spreading solvent). Polar phospholipids, despite being small, are insoluble in aqueous media. The DPPC film generated was more surface active, exhibiting a higher surface pressure of 55mN/m (a remarkable lower dynamic surface tension of only 17.8 mN/m), under maximum compression, in the set minimum volume. A liquid expanded-liquid condensed phase transition is evident (see Figure 6.2 below) in the DPPC film generated. This phase transition is absent in the Langmuir isotherm of the DLPC film (see Figure 6.1). DPPC is more surface active, due to the more saturated C16 molecule being able to pack more efficiently at the air-water interface. The DPPC is of higher molecular weight and hence possesses a greater capability of resisting compression at the air-water interface.

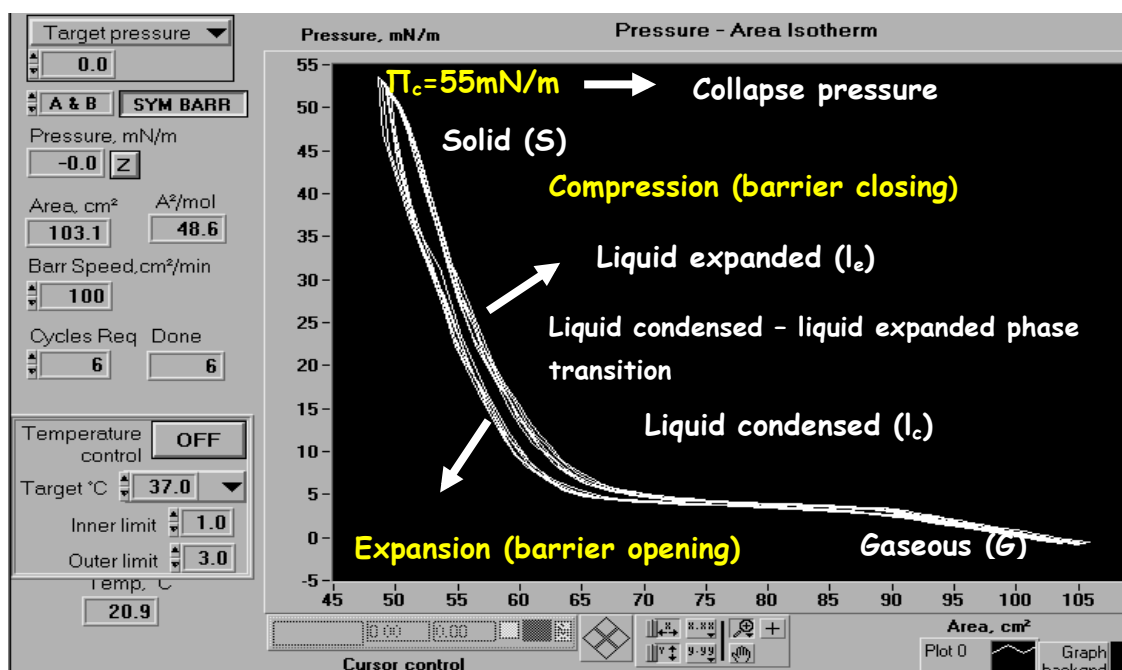


Figure 6.2. A Langmuir isotherm of 1% (w/v) DPPC in  $\text{CHCl}_3$  spread onto an air-water interface, exhibiting a remarkable high collapse pressure, of 55mN/m at 20 °C, compressed at a rate of 100cm<sup>2</sup>/min, demonstrating a distinct phase transition.

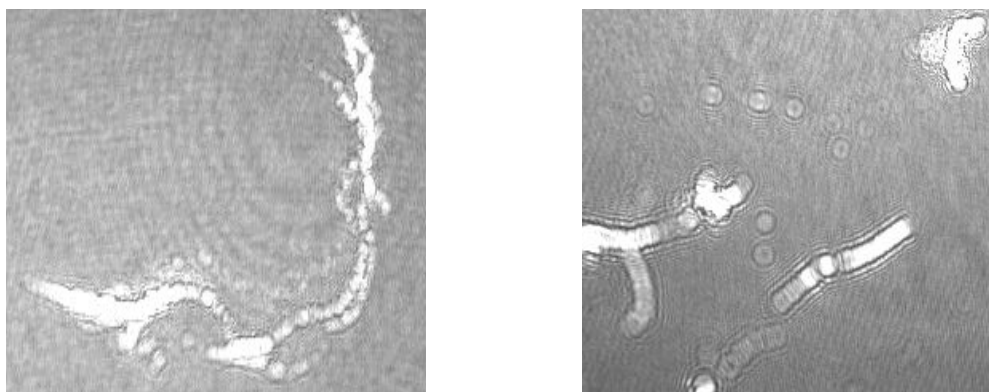


Figure 6.3. BAM images of DPPC film reflections, at air-water interface (visible microstructures - width of image at 100 microns).

## 6.2 Commercial lipid supplementation

### 6.2.1 Clarymist™: A liposome spray to manage lipid deficient dry eye

Clarymist™ liposome spray is an ocular spray with vitamin A & E, for the treatment and relief of dry eyes symptoms. It is effective in the delivery of minute liposomes. One spray claims to apply millions of the small particles to the eyelid. They make the skin moist, soft and supple, lower the temperature of the eyelid and soothe the uncomfortable effects of dry eye; it makes the wearing of contact lenses more comfortable. Liposomes penetrate quickly into the skin providing active hydration, phospholipids and essential fatty acids (linoleic and linolenic acids), vitamin E & A. They ease itchiness and have pain relieving and anti-inflammatory properties. The eyes and eyelids are soothed, irritation reduces and proper functioning restored. Clarymist™ liposome spray stabilises the lipid layer of the tear film and regulates and improves the hydration of the eye surface and the eyelids. It should be used for environment related disorders such as dry eyes, strained eyelids and burning or itching sensation of the eyes.

Table 6.1. Composition of Clarymist™ liposomal spray.

<b><i>Component</i></b>	<b><i>Function</i></b>	<b><i>Concentration</i></b>
soy lecithin	lipid	1%
sodium chloride	salt	0.8%
ethanol	solvent	0.8%
vitamin A palmitate	antioxidant	0.025%
vitamin E (tocopherol)	antioxidant	0.025%
phenoxyethanol	preservative	0.5%

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Figure 6.4, shows the Langmuir isotherm of Clarymist™ liposomal spray film. The Clarymist™ liposomal contents were deposited onto a prior cleaned HPLC grade water subphase surface, in the trough (at 24.4°C) using 25 spray jets to maximise surface coverage. The Clarymist™ liposomal film was left for about 30 minutes, to ensure sufficient time for solvent evaporation; namely ethanol and phenoxyethanol. The Langmuir isotherm of the Clarymist™ liposomal film was collected, and the data obtained implies that it possesses boundary lubrication capability (adsorbs at the air-water interface). The features of the isotherm exhibit hysteresis, a collapse pressure of 33mN/m, and a dynamic surface tension of 39mN/m, under maximum compression, in the set minimum area. The liquid condensed phase is more distinct at 24.4°C. Nevertheless, Clarymist™ liposomal film demonstrates boundary lubricating capability even at room temperature.

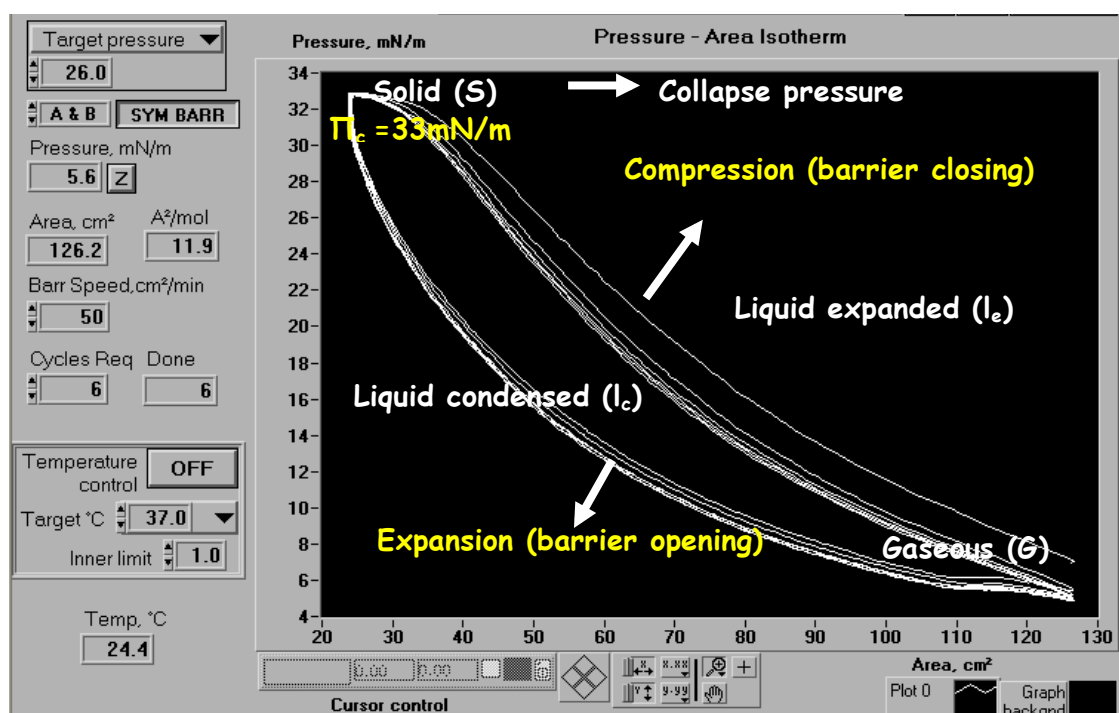


Figure 6.4. A Langmuir isotherm of the Clarymist™ liposomal eye spray at 24.4°C, 25 sprays spread at the air-water interface, exhibiting hysteresis and a collapse pressure of 33mN/m, a dynamic surface tension of 39.8mN/m.

Lipoidal material has different phase temperatures, so the surface activity of the Clarymist™ liposomal film was also assessed at 30°C. The rise in temperature altered the shape and features of the Langmuir isotherm collected, as expected. The collapse pressure, reduced from 33mN/m to 22mN/m as a consequence of the rise in temperature. Concurrently, the dynamic surface tension rose from 39.8mN/m to 50mN/m. This implied that the Clarymist™ liposomal film spray was less stable at higher temperature, due to a lower collapse pressure attained (this indicates that the



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lipid film generated at the air-water is less stable at 30.1<sup>0</sup>C). A lot more hysteresis is apparent in the Langmuir isotherm at 30.1<sup>0</sup>C; the liquid expanded phase transition is extremely evident as opposed to the liquid condensed phase. The greater hysteresis may also be explained by the fact the soy lecithin molecules are more flexible at higher temperature and can spread more readily, i.e., have a greater spreading capability.

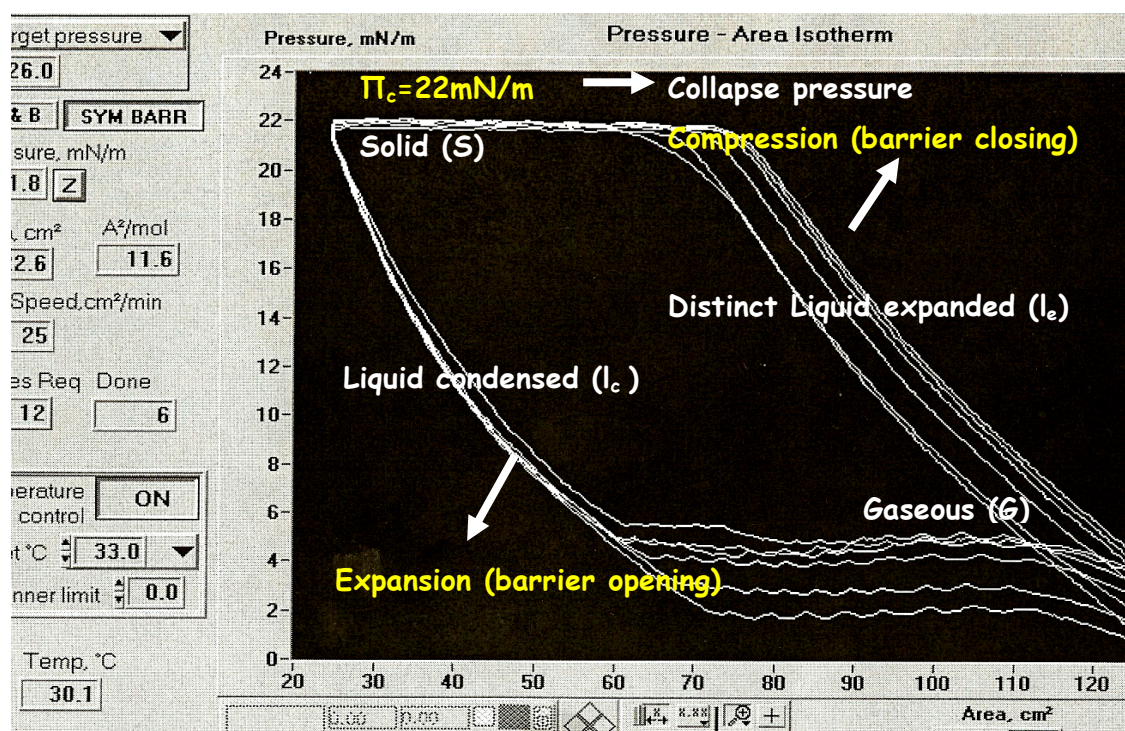


Figure 6.5. A Langmuir isotherm of Clarymist<sup>TM</sup> liposomal spray spread with 25 sprays on the water subphase, at 30<sup>0</sup>C.

### 6.2.2 The surface properties of an ocular Lipoidal film

Extracted ocular Lipoidal material (in chloroform) from a worn balafilcon A contact lens was deposited onto a prior cleaned, water subphase surface. The surface pressure was measured using a Wilhelmy balance pressure sensor, employing a Wilhelmy paper plate, as the film was compressed (squeezed) and expanded (relaxed) - mimicking a blinking action. Brewster angle microscopy (BAM) allowed the visualisation of the Lipoidal film at the air-water subphase surface. Figure 6.6, demonstrates that the native Lipoidal film is very surface active, in that it exhibits a collapse pressure of 55mN/m, and a dynamic surface tension of 17.8mN/m, under maximum compression. No other commercial lubricant behaves in such a manner. The shape of the isotherm is highly expanded, i.e., there is ample hysteresis. A



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distinct liquid expanded phase is present, demonstrating that the Lipoidal film is resisting compression, i.e., the film wants to remain at the air-aqueous subphase surface and oppose the occurrence of collapse. This is strong evidence that the Lipoidal film is in fact the boundary lubricant and therefore has the capability of protecting the ocular surface from the mechanical action of the eyelids during blinking.

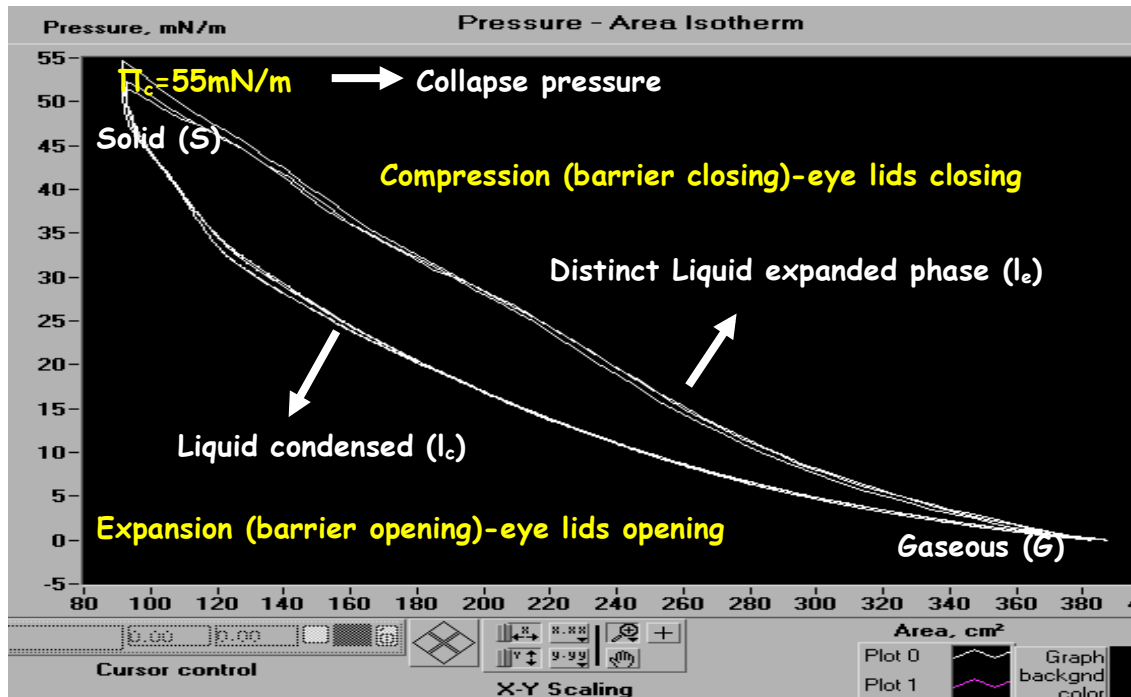


Figure 6.6. Langmuir isotherm of chloroform extracted Lipoidal film from a worn balafilcon A contact lens (after five hours of extraction) showing an amazing high collapse pressure of 55 mN/m, a dynamic surface tension of 17.8mN/m, under maximum compression, in the set minimum area.



Figure 6.7. BAM image, extracted Lipoidal film at the air-water interface from worn balafilcon A contact lens, at room temperature.

BAM images confirmed that human Lipoidal extract from a worn balafilcon A contact lens (from this particular patient) generate films (see Figure 6.7 above). The images confirmed that the Lipoidal extract is highly reflective and must float on top of an aqueous subphase surface. Synthetic mimics (biolubricants) should therefore possess similar properties. These findings can be used as target surface properties, for assessing the performance of a potential synthetic biolubricant in order to supplement boundary lubricant deficient sites, and more so for ocular biolubrication applications.

### **6.2.3 Artificial bovine-derived lung surfactant: Survanta®**

Survanta®, an intratracheal suspension is a sterile, non-pyrogenic, pulmonary surfactant intended for intratracheal use only. It is a natural bovine lung extract containing phospholipids, neutral lipids, fatty acids, and surfactant associated proteins to which colfosceril palmitate (dipalmitoylphosphatidylcholine), palmitic acid, and tripalmitin are added to standardise the composition and to mimic surface tension lowering properties of natural lung surfactant. The resulting composition provides 25 mg/mL phospholipids (including 11.0-15.5 mg/mL disaturated phosphatidylcholine), 0.5-1.75 mg/ml triglycerides, 1.4-3.5 mg/mL free fatty acids, and less than 1.0 mg/mL protein. It is suspended in 0.9% sodium chloride solution, and heat-sterilised. It contains no preservatives. Its protein content consists of two

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hydrophobic, low molecular weight, surfactant associated proteins commonly known as SP-B and SP-C. It does not contain the hydrophilic, large molecular weight surfactant associated protein known as SP-A. Each millilitre contains 25 mg of phospholipids. It is off-white to light brown liquid supplied in single-use glass vials containing 4 mL (100 mg phospholipids) or 8 mL (200 mg phospholipids).

The polymer phospholipid complexes based from poly(maleic acid-co-styrene) and DLPC/DPPC are very surface active; under repetitive expansion and compression they exhibit a dynamic surface tension similar to that of a lung surfactant ( $<5 \text{ mNm}^{-1}$ ), see Figure 6.8-6.9.



Figure 6.8. Dynamic surface tension verses compression for synthetic PMAS/DLPC (This is work carried by Tonge and Tighe at Aston University).

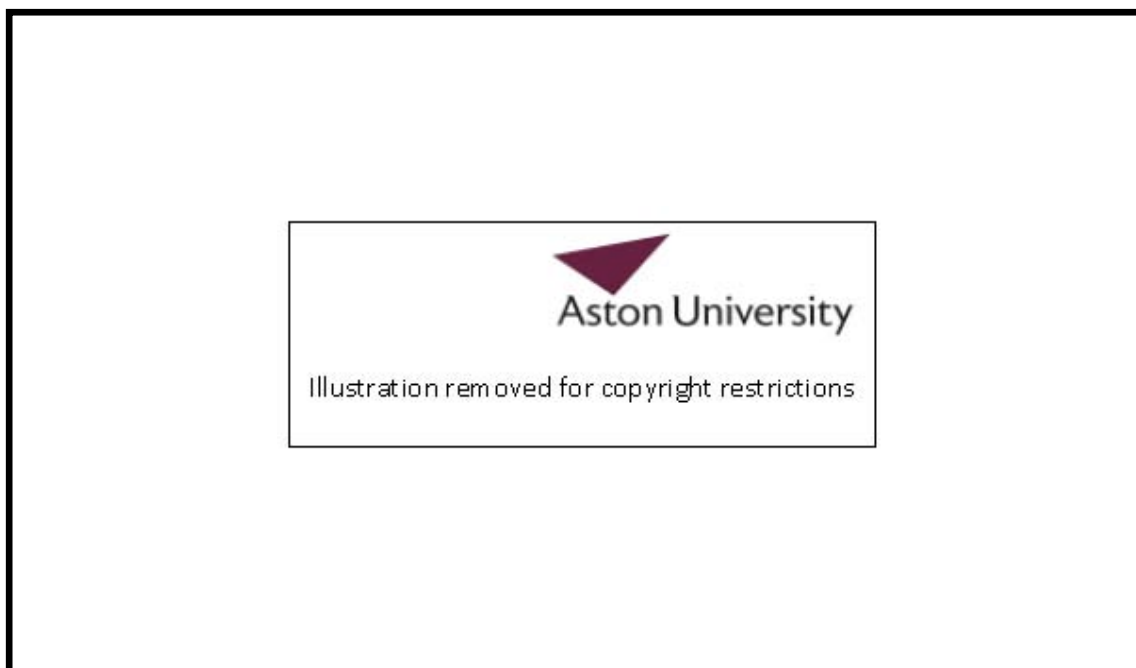


Figure 6.9. Dynamic surface tension vs compression for Survanta® bovine-derived lung surfactant, work carried out by Tighe and Tonge at Aston University) [23].



Figure 6.10. Protein-lipid complexes are present in biological fluids and are observed in Cryo -TEM studies [23].

### **6.3 Biomimetic synthetic biological lubricants: The Aston approach**

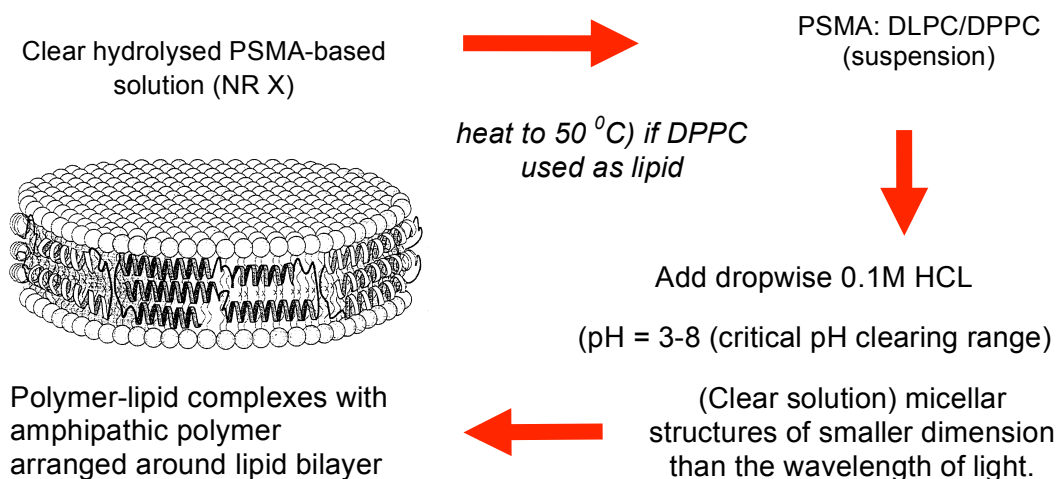
Phospholipids are insoluble in aqueous media it is therefore difficult to design biomaterials from phospholipids, without the use of solvents. Solvents are known to have major disadvantages, for example spreading issues, toxicity and allergy risks. We have designed polymer-lipid complexes that mimic protein-based lipid

assemblies found at fluid interfaces within the body. These materials possess similar functions to native lipoproteins. We have synthesised a range of synthetic apoproteins based from copolymers of maleic anhydride and styrene. These polymers possess analogous (biomimetic) secondary structures to those of the native apoproteins surfactant proteins. The technology developed at Aston provides a means of solubilising and delivering phospholipids to phospholipid deficient sites, without the use of solvents. Our approach has been to mimic the way nature uses proteins to carry lipids and render them water soluble. We have prefabricated apoprotein-lipid complexes analogous to that observed in nature. These materials mimic function of native protein-lipid nanostructures that render biosurfaces lubricious, wettable and at the same time impart a low surface tension (minimise surface forces). Tears, lung surfactant and synovial fluid have highly effective spreading, wetting and lubrication properties, which depend on similar structures. Artificial fluids need the same performance to be truly biomimetic. This is an example of what is now called bionanotechnology, the practical applications of the emerging science of nanotechnology to the area of human medicine.

### **6.3.1 Synthesis of hydrolysed PSMA based-DLPC/DPPC complexes**

The biosurfactants developed in this thesis are lipid-containing compositions, which consists of a substantially clear aqueous solution of a membrane-forming polar lipid and a synthetic amphipathic polymer. This synthetic amphipathic polymer possesses both hydrophobic (styrene/partial esters) and anionic hydrophilic (maleic acid) groups, acting as a lipid-solubilising agent. This amphipathic polymer interact with the polar phospholipid and solubilises it in aqueous medium (at least over a particular pH range). The lipid solubilising synthetic amphipathic polymers specified will generally adopt a helical configuration with the hydrophobic side groups presented along the opposite facet, and that they interact with the lipid in the aqueous medium to form discoidal micellar particles or assemblies of sub-liposomal dimensions in which the lipid forms a bilayer core. See Scheme 6.1, for the picture of polymer-lipid complexes with amphipathic polymer arranged around a lipid bilayer.

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Scheme 6.1. Synthesis of biosurfactants (hydrolysed PSMA-based copolymer-DLPC/DPPC complexes) [1, 23, 26].

Scheme 6.1, above illustrates the synthesis route for the biosurfactants developed in this study. The biosurfactants are prepared from mixing the hydrolysed PSMA-based solution (NR series, see Table 5.4 in Chapter 5, for all PSMA-based solutions used) and the polar lipid of choice (DLPC or DPPC) in the aqueous medium and by adjusting the pH and temperature to effect solubilisation. At a pH above the critical solubilising value (dependent of the hydrolysed PSMA-based copolymer used) and at a temperature, which is above a predetermined phase transition temperature (dependent on the polar phospholipid is used, the temperature region at which the respected polar phospholipid solubilise in solution). The pH can then be adjusted to the desired pH range [1,23, 26]. When a cloudy emulsion of a polymer and lipid is prepared at relatively high pH (such that the polymer is highly charged and most likely in the form of an extended chain), and the pH is then subsequently lowered to a level where the hydrophobic/hydrophilic balance in the polymer chain is suitable for the formation of macromolecular assemblies (this pH level is referred to as the critical pH) a noticeable solubilisation of lipid may be seen to occur which, depending on the quantities and exact nature of the individual components present, results in a marked partial or incomplete clearing of the mixture. The critical pH refers to the pH level below which macromolecular assemblies may form. Styrene-maleic acid copolymers have different critical pH values, depending on their monomer ratios and levels of modifications (esterification levels). Biosurfactants can be synthesised from all 14 hydrolysed PSMA-based copolymer solutions prepared in this thesis (NR1 - NR14, see Table 5.4 in Chapter 5 for further details). The benefits of using lipid solubilising

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agents such as, NR3, NR4, NR6, NR11 and NR12 are that they have a higher hydrophobic component ( $HLB < 20$ ) and hence will form clear solution in combination with a polar phospholipid at a higher pH, and thus likely to be more stable at physiological pH. Additionally, they can load more hydrophobic matter (polar phospholipid) and due to their lower HLB values enhance the surface activity of the biosurfactants. Higher styrene content PSMA-based hydrolysed solutions have higher pH critical values. The pH at which lipid interaction occurs is mainly dependant upon attainment at a particular hydrophilic/hydrophobic balance. PSMA-based copolymers with higher styrene and partial ester groups (higher hydrophobic component) possess less hydrophilic maleic acid groups to neutralise before the correct hydrophilic/hydrophobic balance can be attained, i.e., the balance is attained at higher pH. It is thus possible to tailor the ratio of styrene/partial ester-to-maleic acid ratio such that the polymer interacts with a lipid over a specific pH range, thereby allowing the selection of a particular PSMA-based copolymer, which is ideally suited for a chosen application [1,23-26, 113-115].

HA and co-surfactants (such as Pluronic NF127, a FDA approved biosurfactant) are easily incorporated into all the biosurfactants developed (if desired) for specific application requirements in order to enhance surface activity performance and prolong shelf life, if required.

### **6.3.2 Stabilisation techniques and employment of HA**

#### **6.3.2.1 Freeze drying**

The PSMA-phospholipids have been successfully freeze dried using the method described in Chapter 3, see section 3.7 for detailed methodology employed. Freshly prepared PSMA-DLPC complexes were freeze dried without cryoprotectant.

#### **6.3.2.2 Inclusion of HA into biosurfactant design**

The employment of HA is economically feasible today, because it is readily available from bacterial fermentation processes in a thermally stable form named - HyaCare<sup>®</sup>. The work undertaken in upcoming chapter 7 outlines that the usage of HA in biolubrication applications (e.g., as a biomimetic synthetic synovial fluid) can be optimised, most importantly by combining it with compatible surface active

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components. The main reason being that HA possesses a negligible load-bearing capacity [12]. It has been long suggested that phospholipids are responsible for the boundary lubrication of articular cartilage surface by B.A.Hills *et al* [11]. Thus polar phospholipids are ideal candidates as boundary lubricant agents (surface active components). The challenge has been to combine HA with surface active phospholipids. We have successfully designed lipid solubilising agents (surfactant protein analogues) to form surface active clear solutions. HA can easily be combined with these clear aqueous solutions.

Work reported by Forsey *et al* [53] reports that application of HA and DPPC onto damaged human cartilage resulted in improved lubrication between the cartilages surfaces. SAPLs bind to amino acid groups that comprise the protein chains in proteoglycans such as lubricin. *In-vitro* studies, revealed that the HA molecules adhere to phospholipid membranes (liposome), therefore inhibiting their lysis by  $PL_2$ . This indicates that HA may play an important role in joint lubrication by adhering to SAPLs and protecting the latter against uncontrolled lysis by  $PLA_2$ , which is active in synovial fluid. Their removal induces a significant increase in friction. When functioning under load, the boundary lubrication system adapts itself constantly by a process of remodelling. D.W.Nitzan *et al* [22] concludes that HA inhibits  $PLA_2$ , and thus protects the integrity of the SAPLs .

Contemporary literature suggests that the synergistic effects of the combined HA and Lipoidal complexes (hydrolysed PSMA-based DLPC/DPPC prepared in this thesis), are more effective biosurfactants as opposed to the additive effects of the individual solutions, due to the limitations of the individual solutions [50-53]. Although, HA solutions are good candidates for hydrodynamic lubricants, HA solutions exhibit high surface tension. HA solutions will not remain at the surface of a biological interface. Furthermore, even though hydrolysed PSMA-based-DLPC/DPPC complexes possess amazing surface properties they lack desirable rheological and hydrodynamic frictional properties. These statements are supported by the upcoming experimental findings discussed (see sections 6.4-6.6).

### **6.4 Characterisation of PSMA-DLPC complexes**

This section presents the surface, rheological and frictional properties of PSMA-DLPC/DPPC complexes (for various formulations) both in the absence and presence



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of HA. Particular attention is drawn to the surface properties of the complexes developed, due to the interesting surface properties revealed - as determined via surface measurements. The most intriguing findings were generated from Langmuir and BAM studies, so the use of this technique is the main focus of the experimental results section discussed (see section 6.5-6.6).

### 6.4.1 Static surface tension measurements via du Noüy ring method

The static surface tensions of the freshly prepared PSMA-DLPC were measured using a Pt-Ir (Platinum-Iridium) du Noüy ring. All measurements were recorded to the nearest 0.1mN/m, at room temperature. The static surface tension was measured at least three times to obtain an average static surface tension for the PSMA-DLPC complexes. To assess the viability of the data the standard deviation was also calculated. The Pt-Ir, du Noüy ring was also flamed prior to usage, for each sample measurement. Before each reading was taken, the static surface tension of HPLC grade water was measured (approximately 72mN/m). This was the control/calibration for the experiments. In these experiments the glassware was washed with Fairy Liquid as opposed to Decon 90 (described earlier, in Chapters 3), and left to rinse under hot tap water, for at least 10 minutes. The PSMA-DLPC solutions were allowed to rest before any measurements were undertaken, in case of any foam present at the surface of the solutions. As the presence of foam, effects static surface tension measurements.

Table 6.2. The average static surface tension measurements of biolubricants, measured, via the du Noüy ring dipping method at room temperature.

<b><i>Biolubricant</i></b>	<b><i>Average static ST (mN/m)</i></b>	<b><i>SD</i></b>
HPLC grade water	71.9	0.08
0.1% HA (bacterial fermentation)	57.7	0.52
0.4% Pluronic NF127	42.6	1.44
3% PSMA MW 350, 000 (NR 6)	41.4	0.41
3% PSMA MW 1, 600 (NR 1)	37.8	0.23
3% PSMA MW 1, 900 (NR 7)	35.3	0.05
PSMA-DLPC complex 1 (0.5% DLPC - 1.5% NR 6)	28.2	0.08
PSMA-DLPC complex 2 (0.5% DLPC - 1.5% NR7)	27.3	0.17
PSMA-DLPC complex 3 (0.5% DLPC - 3% NR 1)	26.4	0.08
PSMA-DLPC complex 4 (0.5% DLPC - 3 % NR 6)	25.5	0.17

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The data above clearly implies that the PSMA-DLPC complexes exhibit superior static surface tension values. The water-soluble PSMA-DLPC complexes exhibit remarkable low static surface tensions of  $\leq 28\text{mN/m}$  (25-28mN/m); see Figures 6.12-6.15 for the results obtained in this thesis. The presence of the PSMA-DLPC complexes lowered the surface tension of water, at the air-liquid interface. This demonstrates that the PSMA-DLPC complexes are surface active and adsorb at the air-liquid interface. It must be noted that the slight variations in the static surface tension attained are likely to be due to the difference in the PSMA-DLPC complex compositions, and may also be a consequence of the limitations of the methodology, or possibly due to human error.

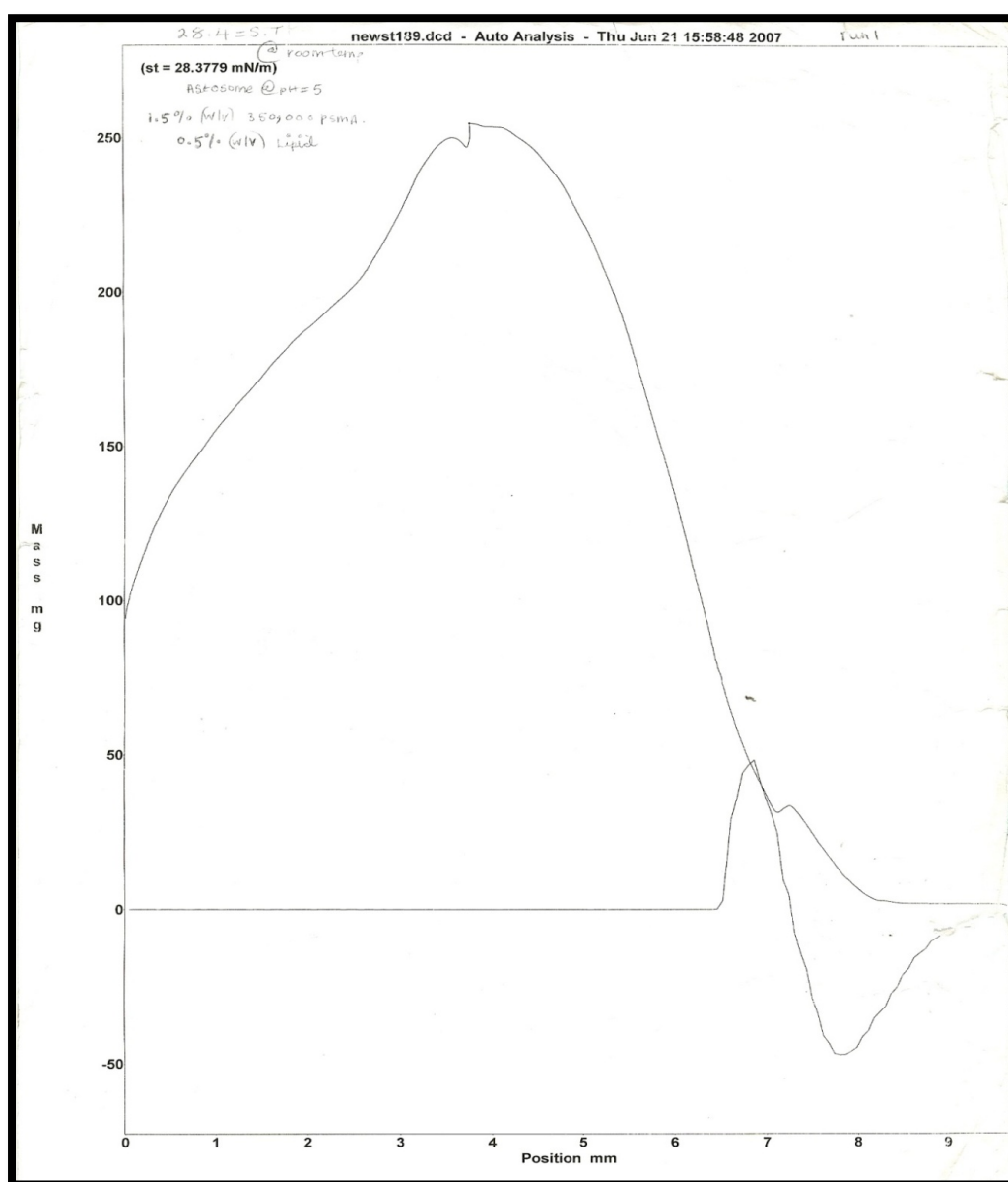


Figure 6.11. Static surface tension measurement of PSMA-DLPC complex (0.5% DLPC - 1.5% PSMA MW 350, 000 - mono-partial methyl ester), exhibiting a static surface tension of 28.4mN/m.

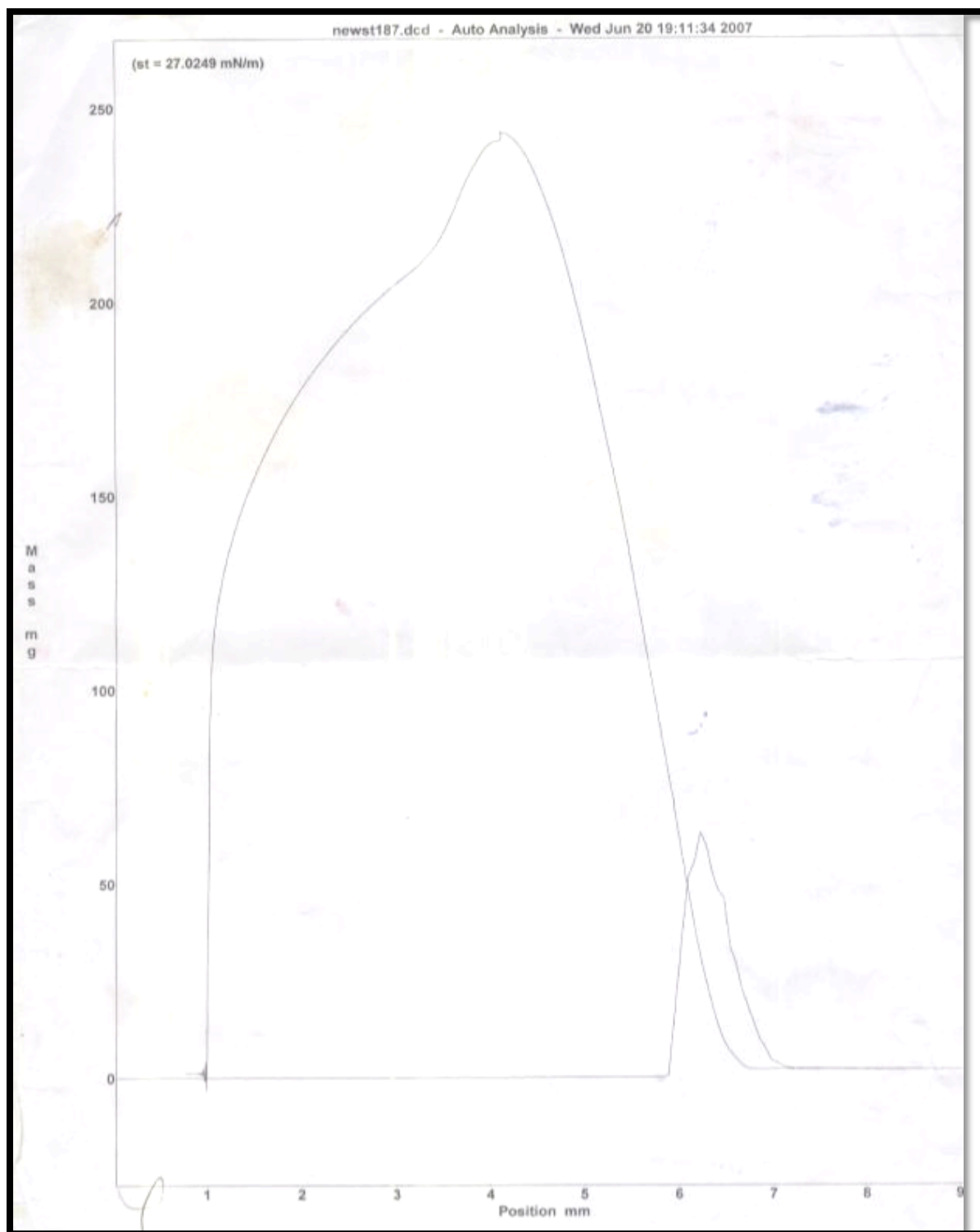


Figure 6.12. Static surface tension measurement of a PSMA-DLPC complex solution (0.5% DLPC - 1.5% PSMA MW 1, 900 - mono-partial propyl ester), exhibiting a static surface tension of 27mN/m.

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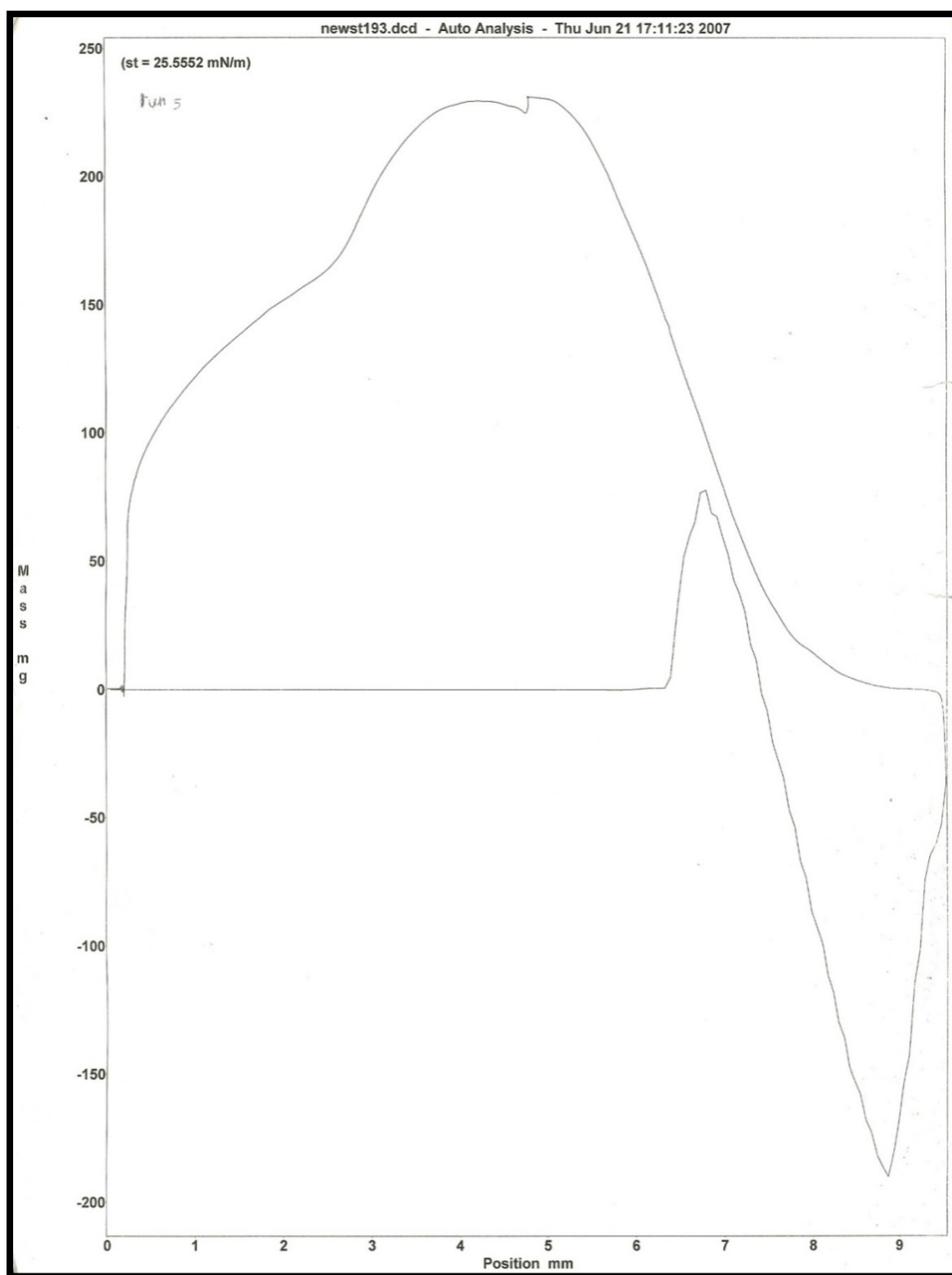


Figure 6.13 Static surface tension measurement of a PSMA-DLPC complex (0.5% DLPC - 3% PSMA MW 1, 600), exhibiting a static surface tension of 25.5mN/m.

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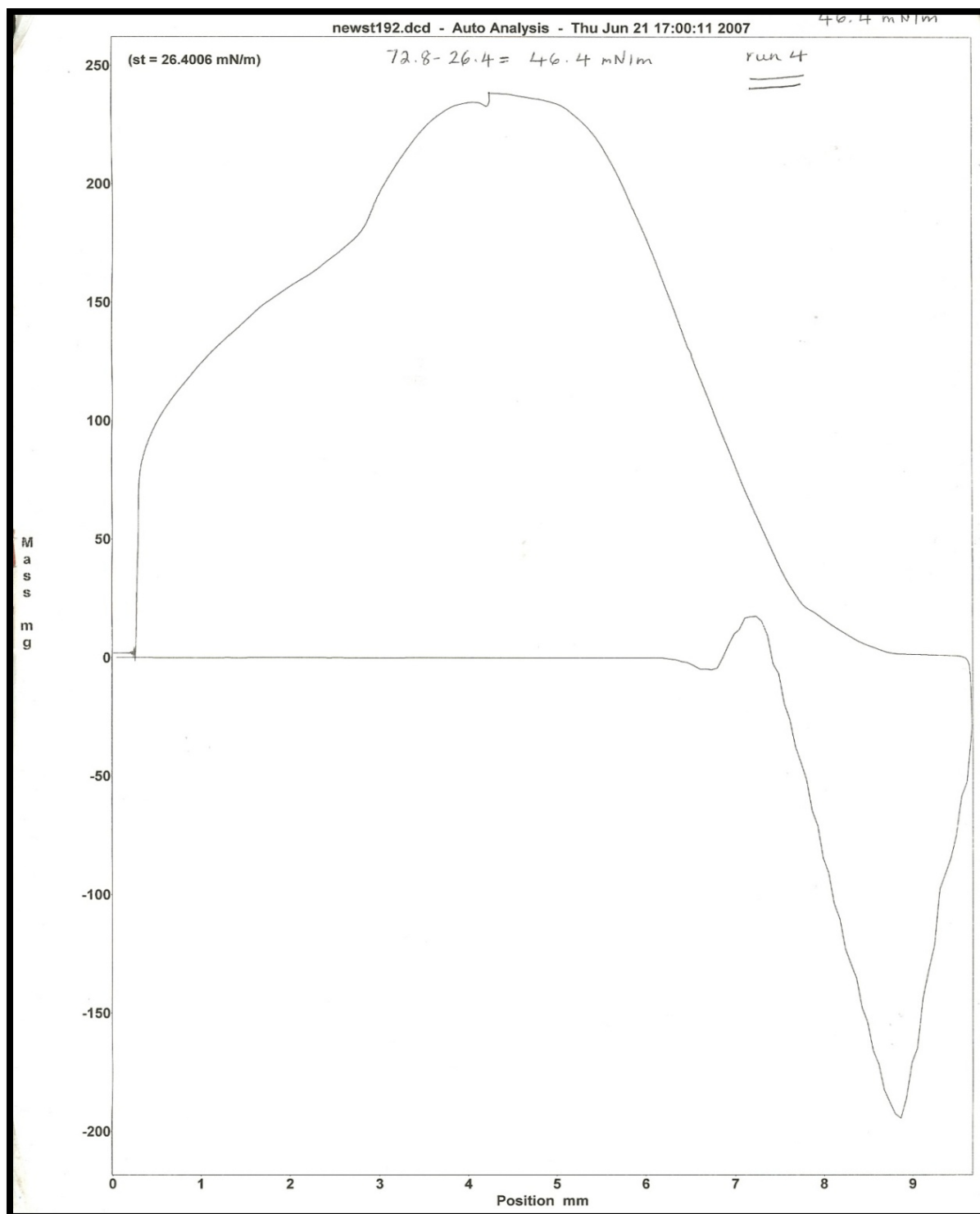


Figure 6.14. Static surface tension measurement of a PSMA-DLPC complex solution (0.5% DLPC - 3% PSMA MW 350, 000 - mono-partial methyl ester), exhibiting a static surface tension of 26.4mN/m.

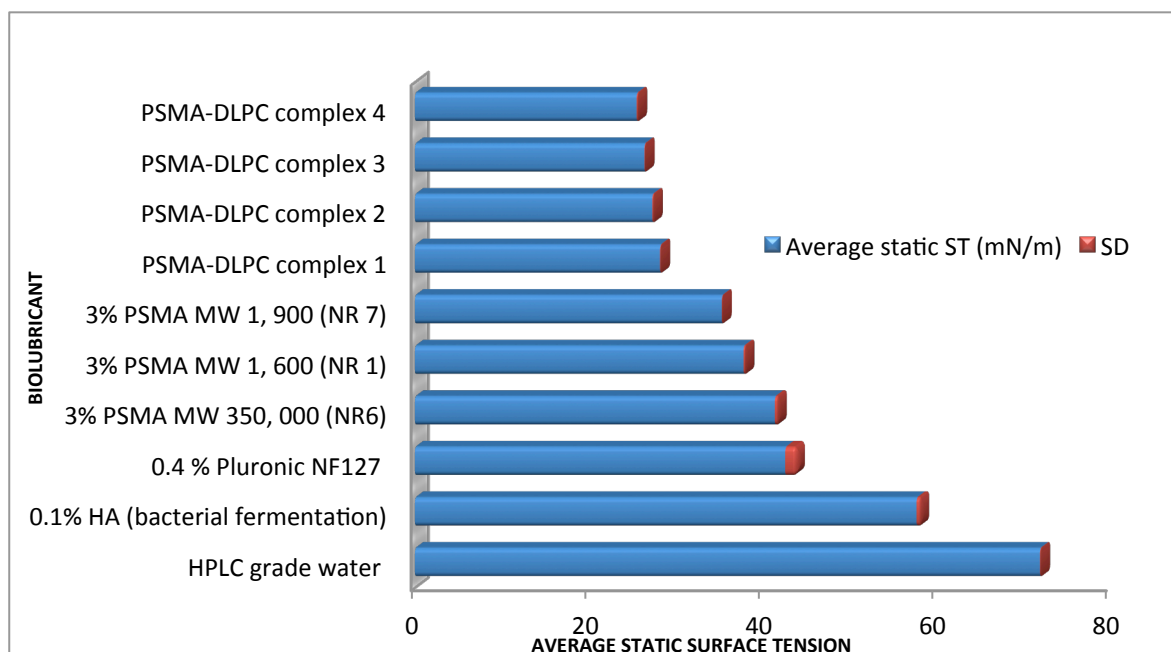


Figure 6.15. The above bar chart illustrates the average static surface tension of biolubricants measured via the du Noüy detachment ring method at room temperature.

Figure 6.15, above depicts that the PSMA-DLPC complexes synthesised in this work have a remarkably low static surface tension ( $\leq 28 \text{ mN/m}$ ). A 0.1% (w/v) HA solution (which is commonly employed as a biolubricant in biomaterials) demonstrates a static surface tension as high as  $58 \text{ mN/m}$  and a dynamic surface tension of  $\sim 65 \text{ mN/m}$  (measured via Langmuir techniques - see Chapter 7 for the Langmuir isotherm of HA). The compositions were selected from those that generated stable clear solutions for the longest duration of time.

Even effective FDA approved ophthalmic employed surfactants, such as Pluronic NF127 (0.4% w/v solution), only demonstrates a static surface tension of  $\sim 43 \text{ mN/m}$ . However, the lipid solubilising agents developed in this thesis, from PSMA MW 1, 600, PSMA MW 1, 900 mono-partial propyl ester 50% (w/v) and PSMA MW 350, 000, 10-15% (w/v) - mono-partial methyl ester, exhibit a lower static surface tension ( $37.8$ ,  $35.3$  and  $41.4 \text{ mN/m}$ , respectively). The most interesting features of these PSMA-DLPC complexes are the remarkable surface properties that they exhibit at the air-water interface. Not only do they have low static surface tensions at the air-liquid interface as measured using the du Noüy method, but they also exhibit high surface pressures-collapse pressures, ( $\geq 45 \text{ mN/m}$ ), and low dynamic surface tensions of  $\leq 27 \text{ mN/m}$ . See sections 6.5-6.6 in this Chapter, for more detailed discussion of the Langmuir isotherms of PSMA-DLPC based complexes. This extraordinary behaviour is not typical for synthetic surfactants. In the case of

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Pluronic NF127 (0.4% w/v), it shows a dynamic surface tension of 52.8mN/m - under maximum compression, in a set minimum area (exhibiting a collapse pressure of ~ 20mN/m via Langmuir technique), despite showing a lower static surface tension of ~ 42.6mN/m, with the use of the du Noüy ring detachment method. The same relationship is also noted for some hydrolysed PSMA-based copolymers already discussed in Chapter 5, with the exception of hydrolysed PSMA MW 350,000, mono-partial methyl ester (10-15%w/v) which possesses both low static and dynamic surface tension values at the air-aqueous interface. The surface measurements (static and dynamic) of the PSMA-DLPC complexes obtained concur well with both techniques; the du Noüy method (static), and the Wilhelmy plate method (dynamic) - via the Langmuir technique. This unique surface behaviour of the PSMA-DLPC based complexes developed in this thesis distinguishes them from all other bio-employed surfactants, which has promising commercialisation potential in the biomaterials sector.

### **6.4.2 Frictional behaviour of PSMA-DLPC/DPPC/HA based complexes**

It is important to measure the coefficient of friction features for the PSMA-DLPC/DPPC complexes in order to assess their lubricating capability. The frictional behaviour of these complexes were conducted using a CSM Nano Scratch Bio-tribometer, with a vibration free table, allowing low coefficients of friction to be measured. The frictional force of the sliding polypropylene head (hydrophobic surface) against the solution (e.g., the PSMA-DLPC complexes, 100 microlitres in this case) was placed onto the Melinex sheet (polyethylene terephthalate) under study, and was recorded as a function of distance travelled in mm (0-20mm). A load of 60mN (normal force) at a speed of 30mm/min was utilised, see section 3.4 (Chapter 3) for further details and the experimental setup. The only variable was the lubricant employed. HPLC grade water was also tested as the control for the experiment. The mean coefficient of friction of the PSMA-DLPC based complexes (0.41) was surprisingly higher than that of the PSMA-DPPC based complexes (0.1); see Table 6.3 for further details of mean data obtained. Such a difference was not anticipated as both DLPC and DPPC molecules both demonstrate boundary lubricating capability, as they are both surface active.

Moreover, combining the PSMA-DLPC with HA (Orthovisc - in this case) lowered the coefficient of friction of the PSMA-DLPC complex (lubricant). It was also interesting

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to observe that the DLPC and DPPC insoluble solutions in HPLC grade water demonstrate very low coefficients of friction ( $\sim 0.06$  - see Table 6.3). A simple explanation for this is that the phospholipids may coat the substrate and show good boundary lubrication. Ultimately, these simple experiments highlight that although PSMA-DLPC complexes exhibit remarkable surface properties at the air-water interface, they do not have the sole capability of functioning as an effective lubricant, at the solid-air interface. This may be due to their small size that impedes surface coverage and hence reduces the capability of providing hydrodynamic lubrication. HA is an excellent candidate for incorporating into solutions which consist of small PSMA-DLPC/DPPC complexes (which are surface active and therefore possess boundary lubricating capability). Furthermore, HA is an excellent component for such low viscosity solutions. These *in-vitro* frictional measurements reflect that the combined PSMA-DLPC/DPPC and commercially available HA (Orthovisc) solutions provide a balance of surface and frictional properties. This combination is favourable as it allows the PSMA-DLPC/DPPC and HA solution to function in the mixed lubrication regime, as a hydrodynamic (provided by the HA component) and boundary lubricant (provided by the PSMA-phospholipid complexes). These features are necessary in order to achieve lower coefficients of friction for effective biolubrication function. The complex solubilises the DLPC/DPPC and allows it to be delivered as a complex lubricant, in a way that is not possible with the insoluble DLPC/DPPC. To test the combined properties further work is necessary for developing a method that breaks down the hydrodynamic layer, to generate the boundary layer.

Table 6.3. Frictional behaviour of PSMA-DLPC/DPPC complexes in the absence and presence of HA (Orthovisc), note coefficient of friction for Orthovisc is 0.09.

<b>Frictional measurement</b>	<b>Biolubricant</b>				
	<b>PSMA - DLPC/HA</b>	<b>PSMA - DLPC</b>	<b>PSMA- DPPC</b>	<b>1% DLPC</b>	<b>1% DPPC</b>
static (1)	0.12	0.41	0.02	0.07	0.06
dynamic (2)	0.12	0.41	0.08	0.09	0.08
static/dynamic (3)	0.98	0.98	0.25	0.78	0.78
mean (4)	<u>0.12</u>	<u>0.41</u>	<u>0.13</u>	<u>0.08</u>	<u>0.07</u>
SD (5)	0.03	0.02	0.08	0.01	0.01



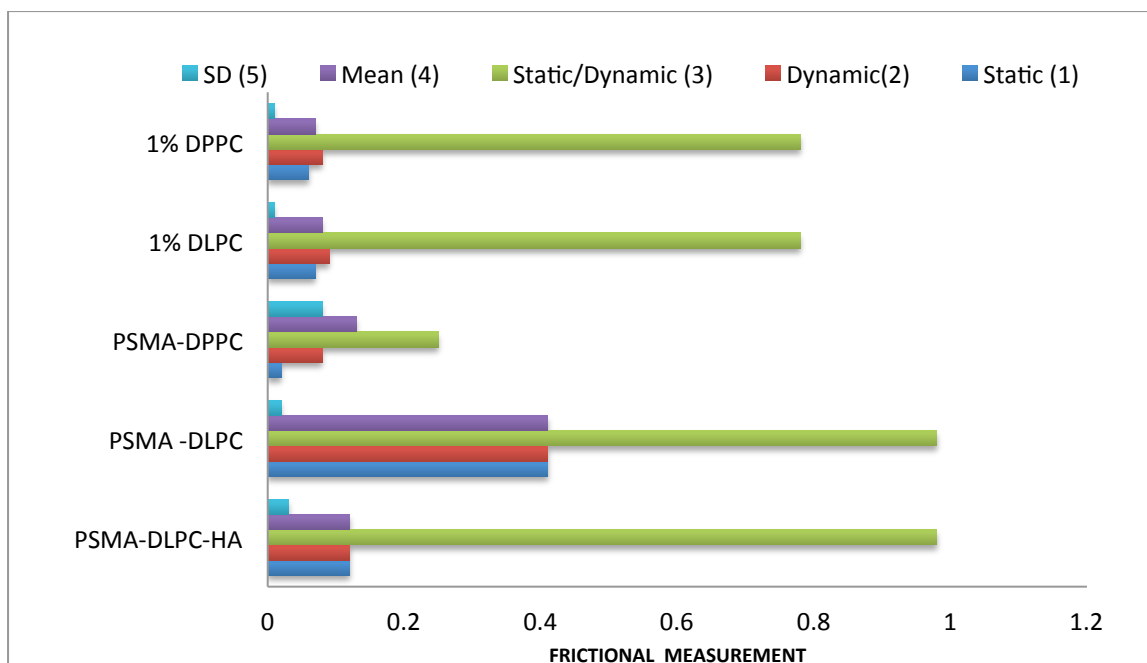


Figure 6.16. Frictional measurements of biolubricants (as specified in Table 6.3) in order; static (blue), dynamic (red), static/dynamic ratio (green), mean coefficient of friction (purple) and the standard deviation (aqua blue).

#### 6.4.3 Rheological behaviour of PSMA-DLPC/ HA complexes

It is useful to assess the rheological behaviour of lubricants, especially if they are to function in the hydrodynamic lubrication regime and need to possess longer residence time at the lubricant deficient site for prolonged lubrication effect. This is achieved if biolubricants are viscous (usually of high molecular weight) or exhibit desirable rheological behaviour that mimic the rheological properties of native biolubricants (i.e., pseudo plastic). A few drops of the freshly prepared PSMA-DLPC complexes (with and without incorporated HA), were placed onto the lower plate of a Bohlin CVO50 rheometer and the viscosity was measured as a function of shear stress, using a CP1<sup>o</sup>/20mm upper plate at 34<sup>o</sup>C.

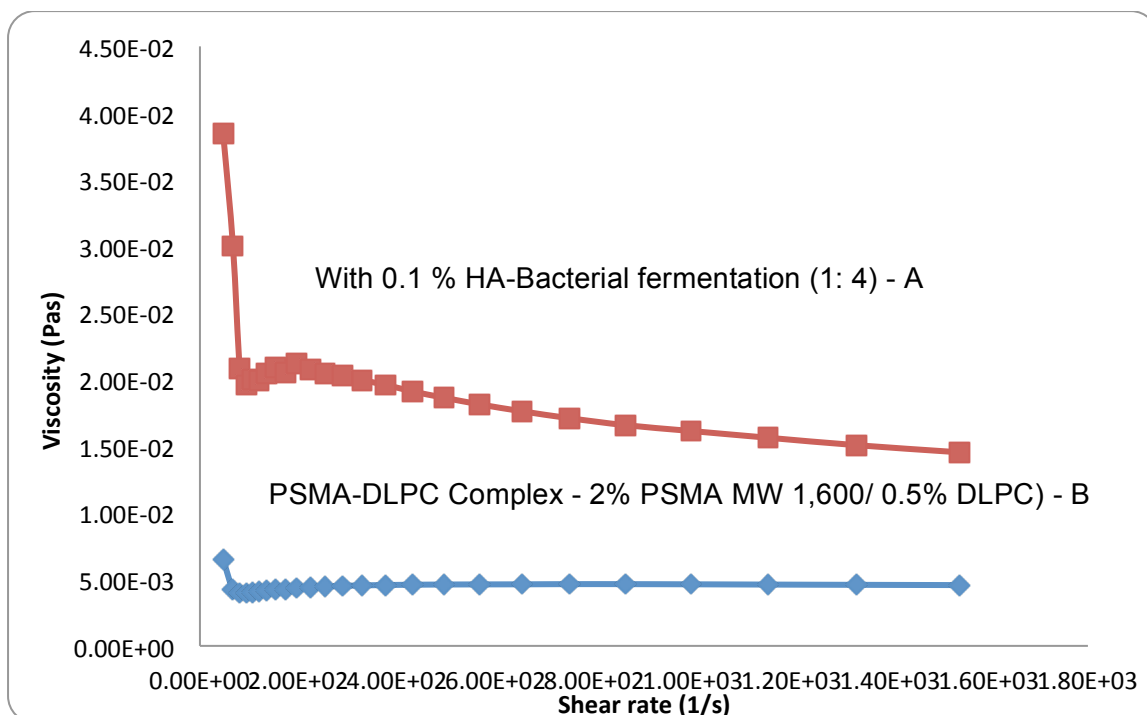


Figure 6.17. The viscosity of PSMA-DLPC: 2%(w/v) PSMA MW 1, 600/0.5% DLPC complex with (A) and without HA 0.1% (B) (w/v), against shear rate.

Figure 6.17 above, depicts that the viscosity of PSMA-DLPC complexes against shear rate. It is apparent from the data illustrated above, that the incorporation of a high molecular weight agent such as HA (in the case here, 0.1% w/v bacterial fermentation sourced) into a PSMA-DLPC solution improves its rheological behaviour. This suggests that that the combined HA and PSMA-DLPC complexes possess rheological properties, that mimic the reported behaviour for native biological lubricants.

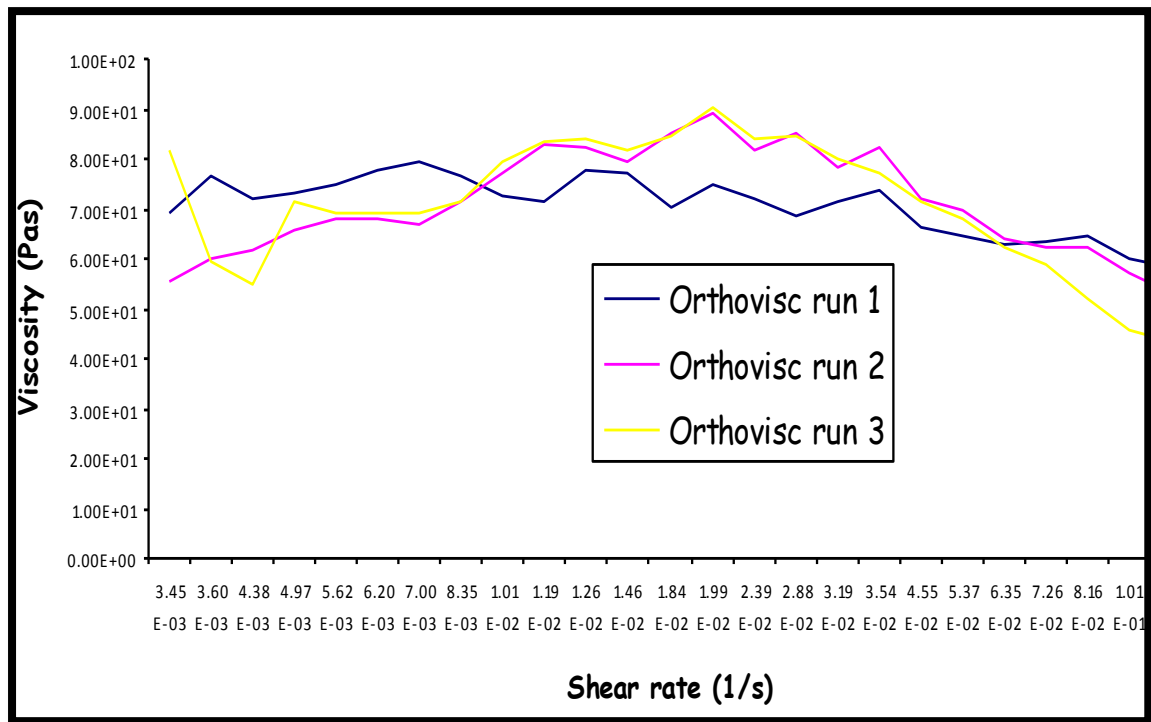


Figure 6.18. The viscosity of 1% (w/v) Orthovisc against shear rate.

Figure 6.18 above reveals that the rheological behaviour of 1% (w/v) Orthovisc, *in-vitro*, is Newtonian. As the shear rate was increased, the viscosity was largely unaffected. Thus, a very viscous solution of HA does not mimic the rheological behaviour of native biolubricants, such as synovial and tears (which are both non Newtonian).

Figure 6.19 shows that the *in-vitro* rheological behaviour of PSMA-DLPC complexes combined with Orthovisc are also non-Newtonian. As the shear rate was increased, the viscosity of the combined Orthovisc and PSMA-DLPC complexes is decreased. This suggests that the combined commercial available HA (Orthovisc) and the PSMA-DLPC complexes developed exhibit non-Newtonian rheological behaviour, that mimics the rheological behaviour of functional native biolubricants, such as tears and synovial fluid.

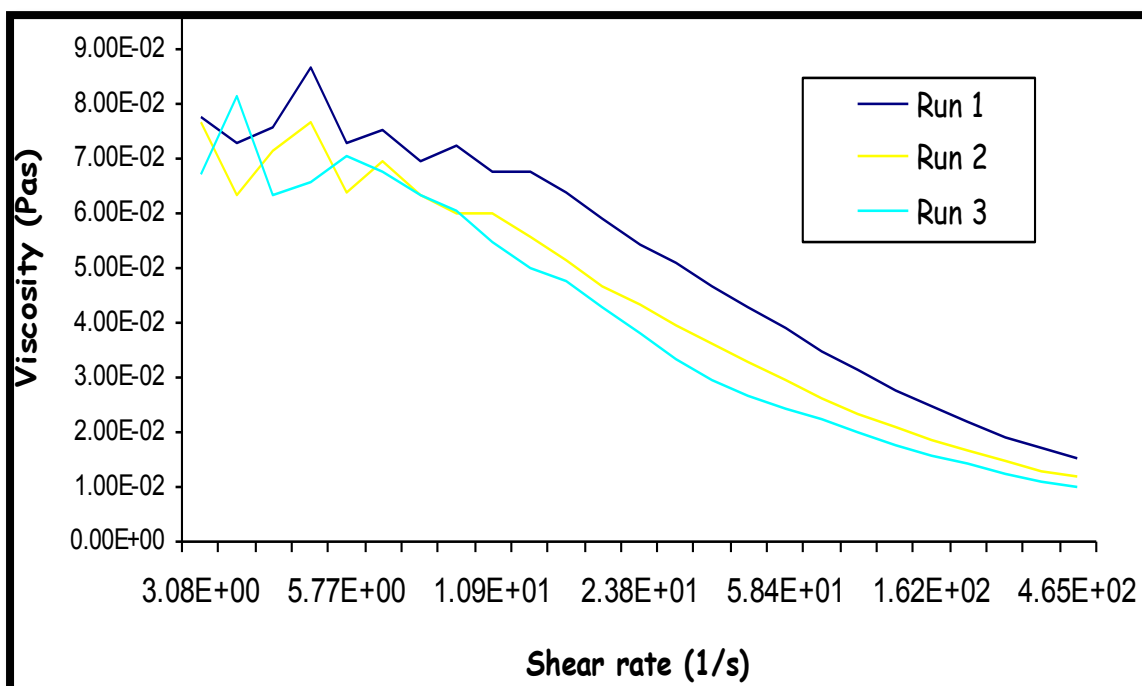


Figure 6.19. The viscosity of Orthovisc / PSMA-DLPC 2% (w/v) PSMA MW 1, 600 - 0.5% DLPC, against shear rate, and Orthovisc at a 4:1 ratio.

## 6.5 Langmuir isotherms of PSMA-DLPC/HA based complexes

A 50 microlitre quantity of the freshly prepared clear solution of PSMA-DLPC, was injected, using a Hamilton syringe (in a dropwise manner), onto the newly cleaned HPLC grade water subphase surface. See Figure 3.18 (Chapter 3) for the Aston experimental setup. Langmuir isotherms of the prepared films of PSMA-DLPC based complexes were obtained at room temperature and under subphase heating to assess their surface behaviour at the air-water interface. Specifically, the rise in surface pressure (reduction in surface tension) was measured as the film (PSMA-DLPC complexes) were repetitively compressed (squeezed) and expanded (relaxed) at room temperature (22.5<sup>0</sup>C), see Figure 6.20.

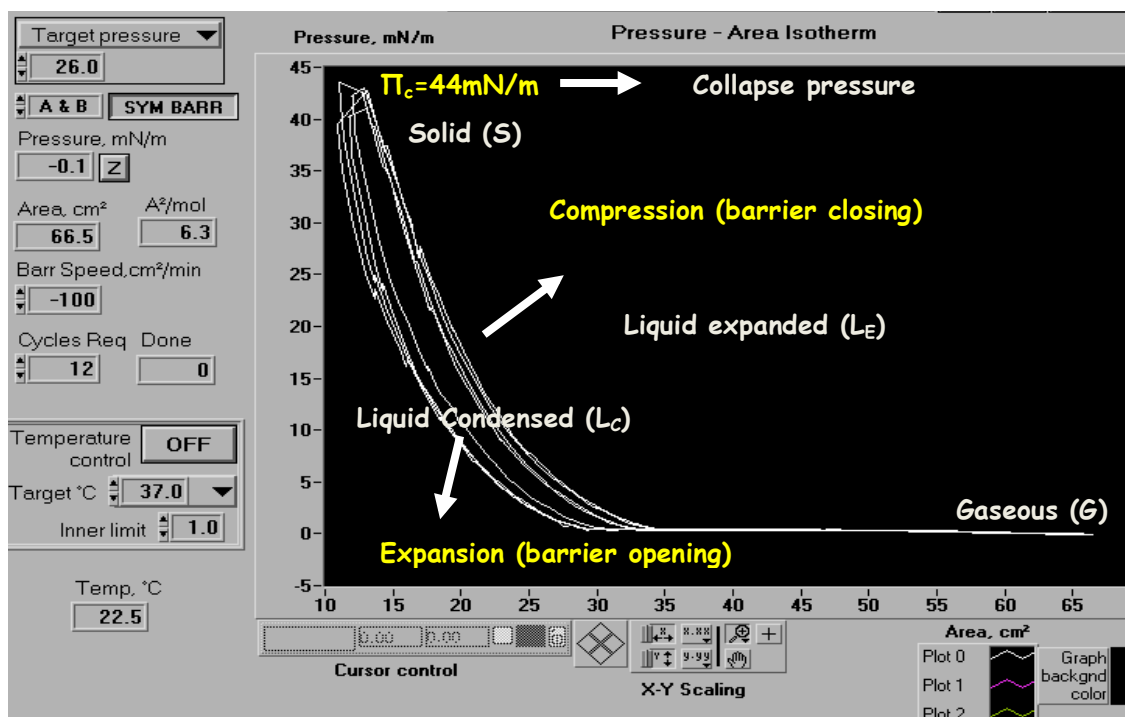


Figure 6.20. A rather condensed Langmuir isotherm of 0.5%DLPC/1.5% PSMA MW 1, 600 (PSMA-DLPC complex) with a collapse pressure of 45mN/m at 22.5 °C, exhibiting surface activity and a high collapse pressure.

Figure 6.20 above, shows the Langmuir isotherm of the PSMA 1, 600 (1.5% w/v) - DLPC (0.5% w/v) formulation. The solution exhibits a high surface activity, a high collapse pressure (44mN/m), i.e., a low dynamic surface tension (28mN/m), under maximum compression, in the set minimum area. This result suggests that PSMA-based lipid solubilising agents have the capability of solubilising, and hence can be used to prepare a surface active, water soluble polar phospholipid solutions, without the usage of solvents. The self assembly (organisation/orientation) of the DLPC with PSMA moieties renders the otherwise water insoluble polar phospholipids aqueous soluble. The hydrophobic styrene groups orientate and interact with the hydrophobic DLPC tails and the hydrophilic maleic acid/maleic acid salt groups interact with the hydrophilic DLPC polar heads and the aqueous media, through hydrogen bonding effects. The amphipathic hydrolysed PSMA molecule orientates itself at the oil (DLPC) water (HPLC water to be more specific) interface. Charge and hydrophobic effect is the principal driving force in the formation of protein lipid-based assemblies (such as those in biological membranes and in defining the conformation of native proteins). The driving forces for the PSMA-DLPC assemblies formation is electrostatic repulsion between the charged side groups, hydrophobic interaction, Van der Waals cohesion between uncharged subgroups, electrostatic attraction, hydrogen bonding, and also solvent and ion effects.

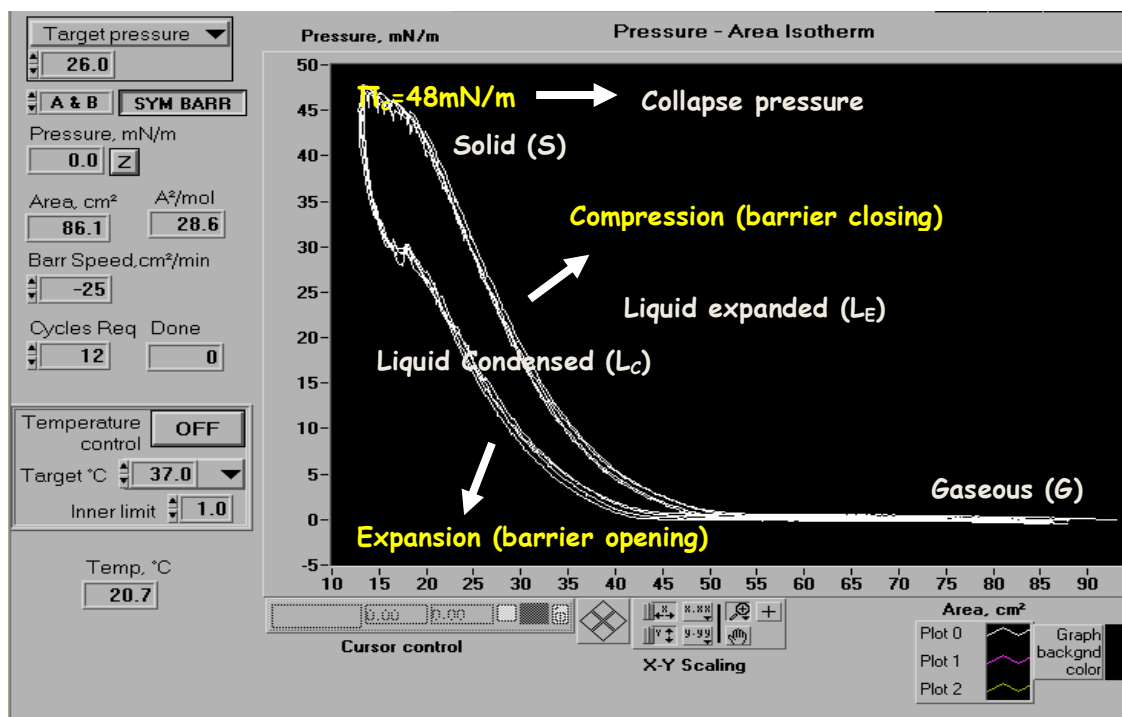


Figure 6.21. A Langmuir isotherm for 0.5% (w/v) DLPC/1.5% (w/v), PSMA MW 1, 600 and 0.4% (w/v) HA (4:1), with a collapse pressure of 48mN/m at 20.7°C, when compressed at a speed of 25cm<sup>2</sup>/min.

Figure 6.21 above, illustrates the Langmuir isotherm of the PSMA MW 1, 600 (1.5% w/v) and DLPC (0.5% w/v) with HA (0.4% w/v solution), as a 4:1 ratio formulation at 20.7°C. This solution exhibits more surface activity (adsorption onto the HPLC grade water subphase surface), a higher collapse pressure (48mN/m) is reached, i.e., a lower dynamic surface tension is reached, under maximum compression (24mN/m). The features of the Langmuir isotherm indicate that presence of HA alters the self assembly of the PSMA-DLPC complex and a PSMA-DLPC-HA complex is generated. The hydrophilic water loving HA may adsorb onto the hydrophilic exposed maleic acid/salt groups that are orientated towards the hydrophilic aqueous water subphase surface. Due to the higher molecular weight of HA, a greater surface coverage is achieved and hence a more stable film is generated at the air-aqueous interface. The data shown in Figure 6.21 also justifies that the incorporation of HA into the DLPC-PSMA formulation is compatible and thus enhances adsorption capability at the air-water interface.

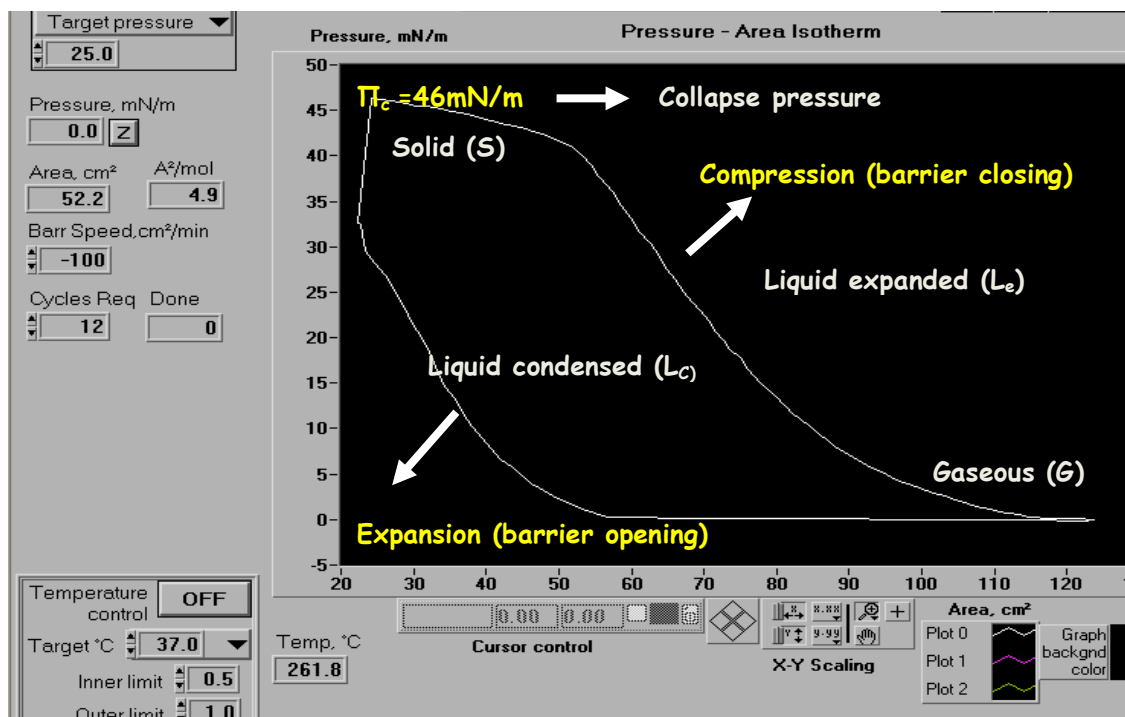


Figure 6.22. A Langmuir isotherm for 0.5% (w/v) DLPC/3% (w/v), PSMA MW 1, 600, with a collapse pressure of 46mN/m, at room temp, exhibiting surface activity when compressed at a speed of 100cm<sup>2</sup>/min.

Figure 6.22 above, shows the Langmuir isotherm for 0.5% (w/v) DLPC - 3% (w/v) PSMA MW 1, 600 solution. By increasing the concentration of the lipid solubilising agent (hydrolysed PSMA) from 1.5 to 3% (w/v) the shape of the Langmuir isotherm is altered. A collapse pressure of 46mN/m is reached and a dynamic surface tension of 26mN/m is attained (under maximum compression) in the set minimum area. The liquid expanded phase ( $L_E$ ) is more distinct, as opposed to the liquid condensed phase ( $L_C$ ). This suggests that the solution with a higher concentration of hydrolysed PSMA (lipid solubilising agent) is more resistant to compression. This generates a more stable film at the air-water interface. Additionally, a higher concentration of polymeric component may also lead to greater surface coverage, more surface activity (a higher collapse pressure), and hence a more expanded Langmuir isotherm shape, as can be seen in Figure 6.23.

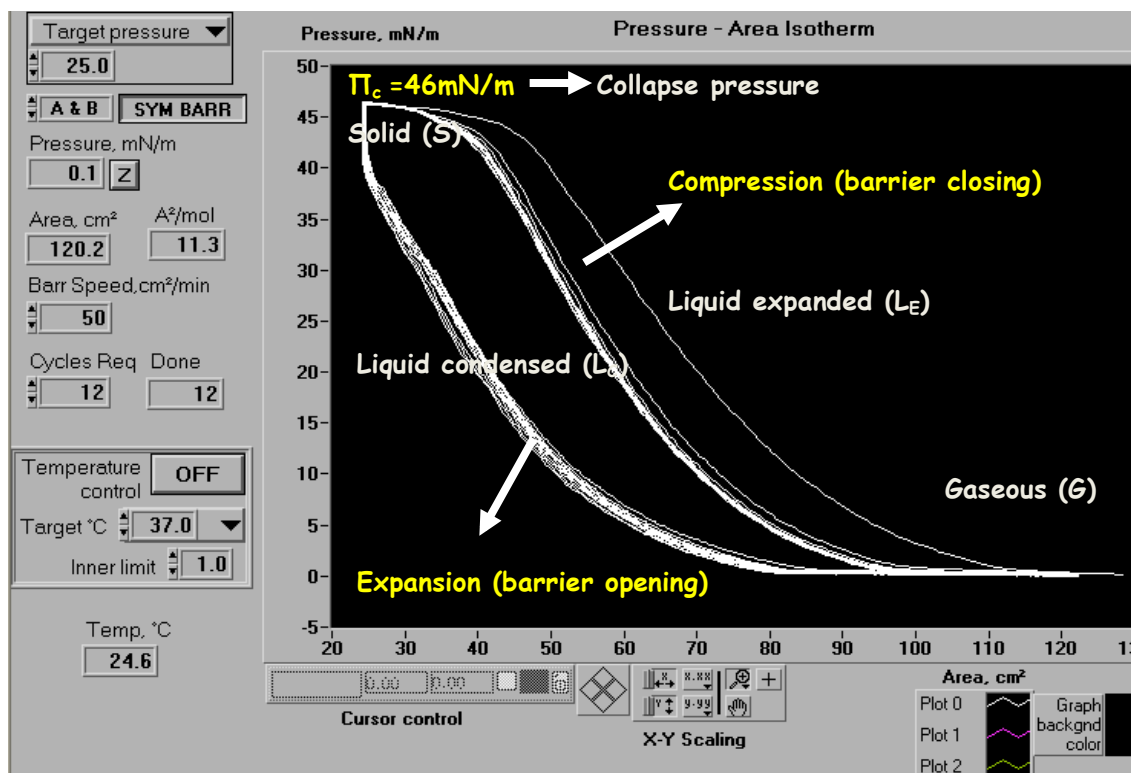


Figure 6.23. A Langmuir isotherm for 0.5%(w/v) DLPC - 1.5%(w/v) blocky PSMA (3:1) MW 9, 500 with a collapse pressure of 46mN/m at 24.6°C, exhibiting surface activity, when compressed at a speed of 50cm<sup>2</sup>/min.

Figure 6.23 above, illustrates the Langmuir isotherm of 0.5% (w/v) DLPC - 1.5 % (w/v) PSMA MW 9, 500 (3:1 monomer feed ratio) based complex, at 24.6°C. A collapse pressure of 46mN/m and a dynamic surface tension of 26mN/m, under maximum compression are achieved (in the set minimum area). A greater hysteresis (a more expanded Langmuir isotherm is generated with the utilisation of a more hydrophobic lipid solubilisation agent (HLB ~ 18.4). The expanded nature may also be due an increase in the molecular weight (greater surface coverage, due to a larger radius of gyration) of the lipid solubilisation agent. The lipid solubilisation agent used is more hydrophobic (HLB ~18.4) in this formulation and may explain why the complex is more surface active. It is more likely to adsorb onto the water subphase surface - due to a greater hydrophobic component orientating towards the hydrophobic air - the surface of the HPLC grade subphase.



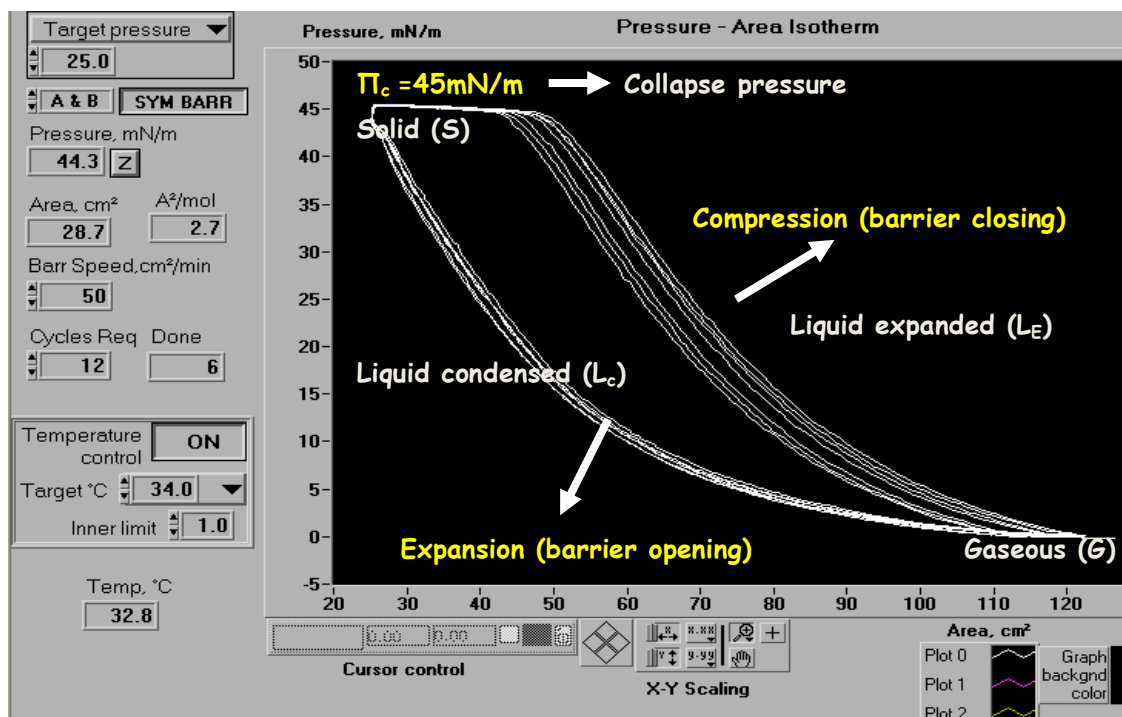


Figure 6.24. A Langmuir isotherm of 0.5% (w/v) DLPC - 1.5% (w/v) blocky PSMA (3:1) MW 9, 500 with a collapse pressure of 45mN/m, at 32.8°C - exhibiting surface activity, when compressed at 50cm<sup>2</sup>/min.

Figure 6.24 above, depicts the Langmuir isotherm for 0.5% (w/v) DLPC - 1.5% (w/v) PSMA MW 9, 500 (3:1 monomer feed ratio), at 32.8°C. At a higher temperature the isotherm shape is more expanded, the collapse pressure reached is attained at 45mNm, a little lower than at room temperature. However, this may be due to experimental error. Nonetheless, a greater hysteresis and a more expanded isotherm is generated as the temperature of the air-water interface approaches physiological temperatures >30°C. The Langmuir isotherm shown in Figure 6.24 indicates that the surface behaviour of the PSMA-DLPC is unaffected by the rise in temperature (approaching physiological temperature). Whereas, the isotherm obtained for Clarymist<sup>TM</sup> liposomal spray film was significantly affected by the rise in temperature and was additionally unstable at around 30°C.

Figure 6.25 illustrates the Langmuir isotherm of PSMA-DLPC complex 0.5% (w/v) DLPC - 3% (w/v) PSMA MW 350, 000, 10-15% (w/v), mono-partial methyl ester. The Langmuir isotherm features for this formulation show a higher collapse pressure (50mN/m), the lowest dynamic surface tension, under maximum compression in the set minimum area.

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The usage of a higher molecular weight lipid solubilising agent (MW 350, 000) which is also more hydrophobic (HLB value  $\sim 17.5$ ) generates a Langmuir isotherm, which is much more expanded. In addition, it possesses an obvious liquid expanded-to-liquid condensed ( $L_E$ - $L_C$ ) face transition. This strongly suggests that the self assembly of the PSMA-DLPC complexes is altered, at the air-water interface and is dictated by the choice and concentration of the lipid solubilisation agent (hydrolysed PSMA-based solution) employed. A higher molecular weight lipid solubilising agent with additional hydrophobic, mono-partial methyl ester groups alters the polymer-phospholipid interactions. The hydrophobic interaction is further enhanced, giving stronger adherence and greater area of contact for the hydrophobic DLPC tails, due to a greater concentration of hydrophobic moieties. This in turn, permits the formulation of a more surface active biolubricant, at the air-water subphase. Therefore, overall the self assembly of the PSMA-DLPC complex is effected by the choice of the lipid solubilising agent.

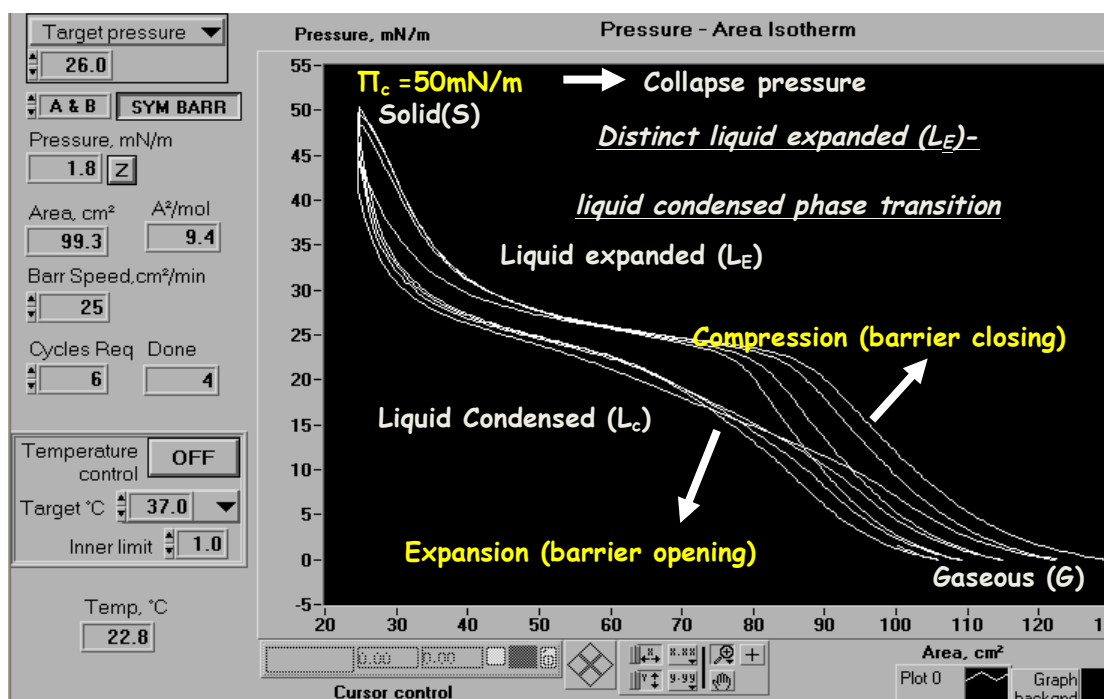


Figure 6.25. A Langmuir isotherm of 0.5% (w/v) DLPC - 3% (w/v) PSMA MW 350, 000 - mono-partial methyl ester (PSMA-DLPC complex) with a collapse pressure of 50mN/m, a dynamic surface tension of 22mNm, exhibiting amazing surface activity, and a distinct  $L_E$ - $L_C$  phase transition at 22.8°C when compressed, at a rate of 25cm<sup>2</sup>/min.

## **6.6 Imaging PSMA-DLPC complexes with Langmuir and BAM**

A *p*-polarized light beam incident on the surface of water at the Brewster Angle is not reflected, but if a monolayer, bilayer or multilayer is spread on the surface it changes the refractive index and some reflection occurs. This principle is used in BAM to observe microscopic structures in a floating film. See Figure 3.17 (in chapter 3) for a discussion of the principles of BAM. BAM was utilised, for the first time to visualise the PSMA-DLPC complexes in order to understand the morphology, size and appearance of the PSMA-phospholipid complexes, under repetitive expansion and compression at the air-water interface. Before any surface imaging can be performed the water subphase needs to be cleaned. To confirm cleanliness, the Langmuir isotherm of the clean subphase is always collected. Figure 3.16, in Chapter 3, shows a typical calibration curve of a clean water subphase surface. A clean water subphase surface always must have a surface pressure less than 1 (a surface tension of about 72mN/m). A clean water subphase can also be confirmed with the use of a BAM, as it will allow visualisation of any dust or contaminants, at the water subphase surface. This was also carried out, so that surface images of the clean water subphase surface concurred with the surface pressure measurements attained; see Figure 3.19 (in chapter 3) for typical images obtained for when the Langmuir/BAM is successfully calibrated.

A freshly prepared PSMA-DLPC complex was prepared from hydrolysed PSMA, MW 9, 500, with a styrene-to-maleic anhydride monomer feed ratio of 3:1. This lipid solubilising agent was deliberately selected due to its estimated HLB value (~14). Figure 6.26 depicts the Langmuir isotherm of this PSMA-DLPC formulation and it reveals that the surface behaviour of this PSMA-DLPC complex is as surface active as the formulation described earlier (see Figure 6.25). A high collapse pressure is obtained (50mN/m) and a low dynamic surface tension - 22mN/m, under maximum compression, in the set minimum area. Additionally, a greater hysteresis is apparent with the use of a more hydrophobic lipid solubilising agent when synthesising PSMA-DLPC complexes. This can provide us with clues as to how different lipid solubilising agents interact with the DLPC molecules at the oil-water/air-water interface. A more hydrophobic lipid solubilisation agent will orientate itself towards the hydrophobic air and the hydrophobic DLPC tails. It will possess greater area of contact (for hydrophobic interaction) with hydrophobic moieties. This in turn, will allow enhanced adsorption and greater hydrophobic interaction, at the air-water interface.

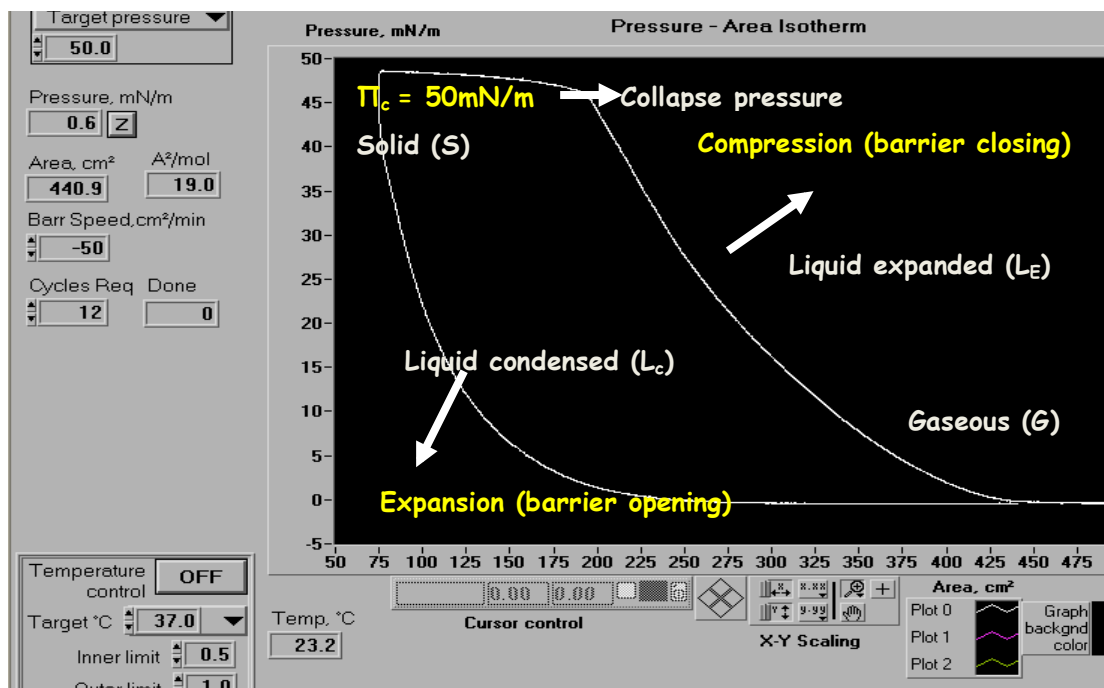


Figure 6.26. A Langmuir isotherm of 0.5% DLPC - 3% (w/v) blocky PSMA (monomer feed ratio of 3:1), MW 9, 500, with a collapse pressure of 50mN/m at 23.2°C, exhibiting considerable surface activity and a high collapse pressure, a highly expanded isotherm, when compressed at a rate of 50cm<sup>2</sup>/min.

Overall, PSMA-DLPC complexes exhibit amazing surface properties that can be optimised by careful selection of the hydrolysed PSMA-based copolymer, and by careful manipulation of the formulation. To further investigate the surface behaviour, BAM was used for the first time to visualise PSMA-DLPC complexes, at the air-water interface. Figure 6.27 shows the BAM images obtained for this PSMA-DLPC type at 23.2 °C. Firstly, it can be confirmed that the PSMA-DLPC complexes can be seen with BAM, which provides strong evidence that they form microstructures as well. A reflection is most definitely present, thus confirming adsorption onto the hydrophilic HPLC grade subphase surface, at the air-water interface. The PSMA-DLPC films must therefore float onto the aqueous subphase surface. Moreover, what is clear from the images obtained is that aggregates (complexes) can be seen and become more defined (clear) under compression. The aggregates are scattered, in the expanded state but cohere together under compression (due to the mechanical action of the trough barriers). This may perhaps be a consequence of forcing the self assembly of the PSMA-DLPC complexes, due to the compressing the PSMA-DLPC floating film (demonstrating a mechanical responsive behaviour too), at the air-water interface.

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Additionally, the PSMA-DLPC complexes formed from the employment of a hydrophilic low molecular weight lipid solubilising agent (PSMA MW 1,600, HLB ~ 28) can also be seen in very similar appearance, on the surface of a pH 7 phosphate buffer subphase, see Figures 6.27-6.28 for images captured.

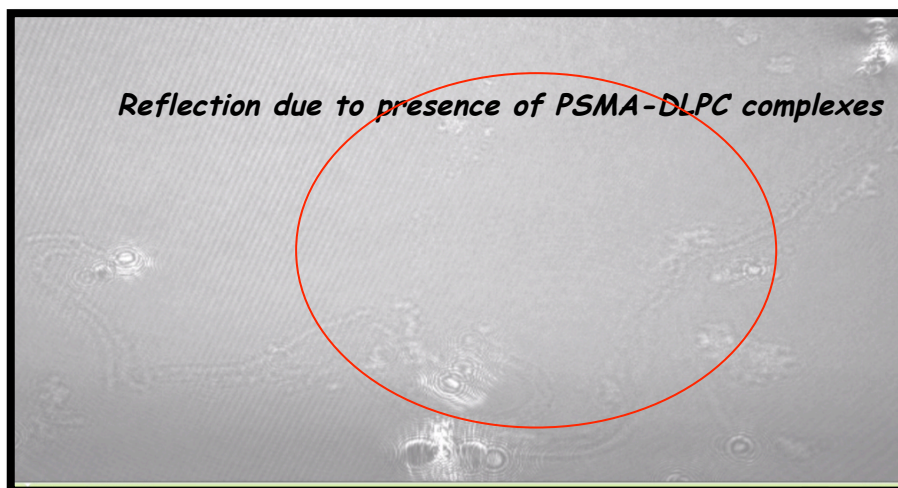


Figure 6.27. A BAM of image of PSMA-DLPC complexes (3% w/v PSMA blocky MW 7, 500, monomer feed ratio 3:1 - 0.5% DLPC), adsorbed at the air-water interface held under compression, showing the visualisation of microstructures (aggregates).



Figure 6.28. A BAM image of PSMA-DLPC complexes (3% w/v MW 1, 600, 1:1 monomer feed ratio - 0.5% DLPC) on phosphate buffer solution, visual aggregates, indicative of microstructures complex formation.

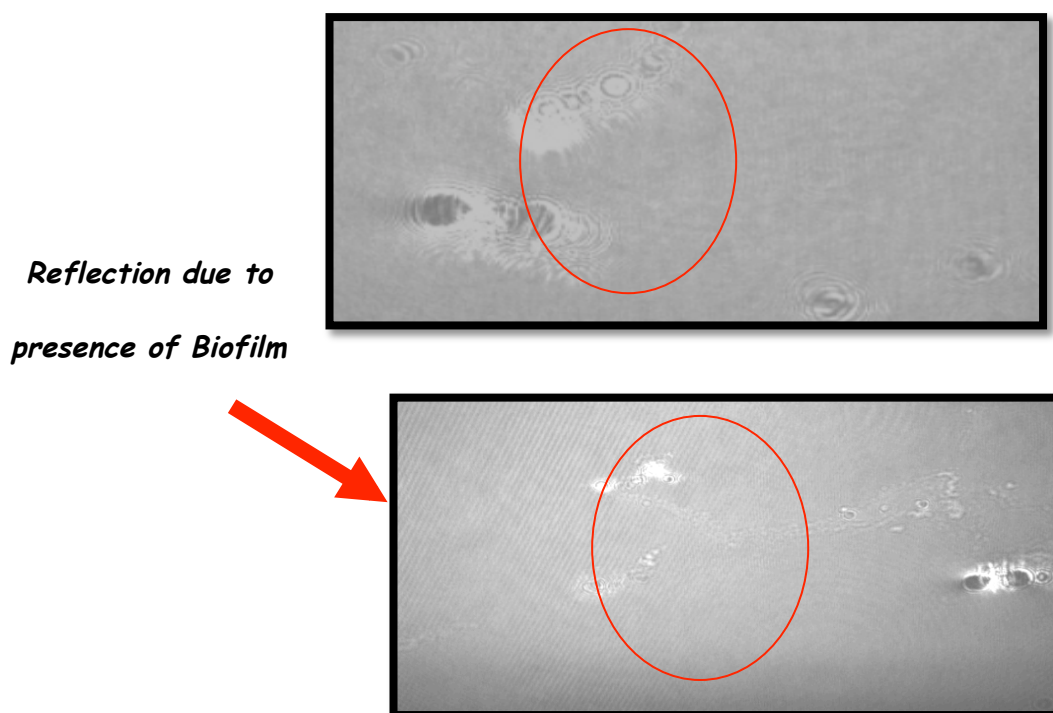


Figure 6.29. Images captured of PSMA-DLPC complexes 0.5 % (w/v) DLPC - 3% (w/v) PSMA MW 9, 500 (3:1 monomer feed ratio).

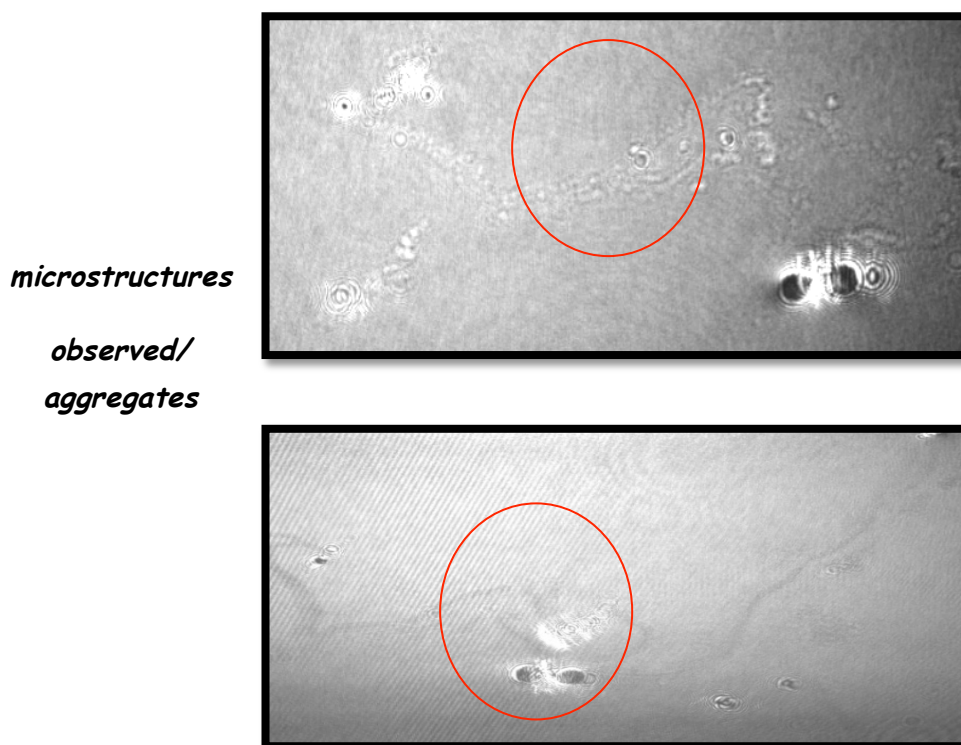


Figure 6.30. BAM images of PSMA-DLPC complexes adsorbed at the air-water interface, held under compression, showing the visualisation of microstructures (as microstructures are only visible via BAM formed at the air-water interface, spread on a prior cleaned HPLC grade water subphase surface).

## **6.7 Discussion**

Due to the range of motion that occurs at the biological surface interface, Lipoidal material plays a crucial protecting role via boundary biolubrication. Lipoidal films are nature's load bearing boundary lubricants and have the capability of adsorbing and retaining at the biological interface. Native lipid is not solely responsible for the remarkable low coefficient of friction exhibited, e.g., at synovial joints. Lipids are insoluble in aqueous media, strong evidence in the literature discusses that the interaction of lipids with proteins in nature renders them soluble in aqueous media, through hydrophobic interactions.

Langmuir isotherms of DLPC and DPPC molecules demonstrate amphipathic behaviour and hence establish that they are excellent candidates for boundary lubricating agents. Polar phospholipids have the capability of adsorbing at the air-aqueous surface. Langmuir experiments provided experimental evidence that the DPPC molecules are more surface active than the DLPC molecules and therefore more effective agents for employment. The challenge to deliver these boundary lubricating agents (without the use of solvents) has been undertaken and achieved. Lipid supplementation has already been commercially undertaken to manage lipid deficient diseases such as dry eye and respiratory distress syndrome but no attempt has been made to manage OA. There is thus a gap in the market for lipid supplementation at articular joints.

Phenoxyethanol (an alcohol based preservative) and ethanol have been utilised to deliver phospholipids to manage lipid deficient dry eye in Clarymist™ liposome spray. An animal based lung surfactant (a mixture of proteins and lipids) known as Servant®, is administered to manage respiratory distress syndrome. These two developments have major drawbacks as the solvent component has toxicity and irritation issues. Additionally, animal derived products have allergy risks.

In this study, synthetic protein-lipid analogues have been successfully synthesised, that mimic the unique surface properties of native biological lubricants without the use of solvents. Extracted native Lipoidal films demonstrate remarkable surface activity, an amazing collapse pressure of 55mN/m - low dynamic surface tension of 17.8 mN/m, under maximum compression in the set minimum area. The BAM images obtained concurred well with the surface numerical values determined. A



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highly reflective homogenous film was revealed. These results can be used as target numerical values, to compare the surface properties of novel ocular synthetic biomimetic lubricants. The data presented in this chapter demonstrates that other commercial attempts such as Clarymist™ liposomal spray and Servant® have not achieved this target surface behaviour.

In this thesis, PSMA-phospholipid complexes have been successfully prepared from a broad range of hydrolysed PSMA-based copolymers (novel lipid solubilising agents) in combination with both DLPC and DPPC. The temperatures employed during synthesis vary due to the differences in the phase temperature of the two phospholipids, the pH clearing range (the critical pH), and depend on the nature of the lipid solubilising agent (hydrolysed PSMA-based solution) employed.

Rheological analysis findings imply that the combined HA and PSMA-DLPC complex exhibit a rheological behaviour that mimics the non-Newtonian behaviour of native biological lubricants.

Frictional measurements demonstrate that the combined HA and PSMA-DLPC complexes exhibit a lower coefficient of friction than the PSMA-DLPC complexes alone.

BAM analysis illustrates that the PSMA-DLPC complexes adsorb and form aggregates at the air-HPLC grade water interface, at room temperature. As the complexes are visible this provides strong evidence that they form microstructure too, as only microstructures are observed via BAM technology. This indicates that the size of the PSMA-DLPC complexes must therefore have a population (range) of micro and nanostructures (as Tighe and Tonge *et al* have reported the complexes to be nanostructure in size via cryo-TEM analysis [23]. During the collection of the isotherm data, it was observed that the microstructure become more evident (more visible via BAM imaging) as the PSMA-DLPC complexes were compressed. This indicates that the PSMA-DLPC complexes are responsive (mechanically) and they may be able to adopt their structure and size, as the self assembly is forced by the mechanical action of the trough barriers, during repetitive expansion and compression. This was an attempt to mimic the mechanical action of breathing, blinking and articular joint movement, as the Lipoidal films collapse and reform at the biological interface surface.



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Langmuir and BAM techniques provide valuable information about the surface properties (spreading, stability and boundary lubrication capability), of biolubricants. In future, this technique has great potential for assessing the effectiveness of boundary lubricants and biological fluids, at the air-aqueous interface.

Additionally, PSMA-DLPC/DPPC complexes can be freeze dried to prolong shelf life and sustain stability and thus have the potential for commercialisation. The PSMA-DLPC based complexes exhibit amazing low static surface tensions (26mN/m) as demonstrated via the du Noüy method and low dynamic surface tensions (at the air-water interface) via Langmuir studies (collapse pressures of >45mN/m were observed). The static surface tension values agreed well with the dynamic surface tension values obtained, via Langmuir studies.

The collapse pressure of the PSMA-DLPC complexes can be enhanced by using higher molecular weight PSMA MW 350, 000 - 10-15% (w/v), mono-partial methyl ester and the blocky (NON-ALT) PSMA MW 9, 500 (St: MA 3:1 monomer feed ratio) lipid solubilising agents. These novel lipid solubilising agents prepared have an estimated HLB value of 17.5 and 14, respectively. The use of higher molecular weight and more hydrophobic lipid solubilising agents enhances the adsorption of the PSMA-DLPC complexes, at the air-water interface. This is evident from the features of Langmuir pressure-area isotherm generated. A greater hysteresis and a higher collapse pressure of 50mN/m are reached, denoting to a dynamic surface tension of 22mN/m under maximum compression (in a set minimum area), as the PSMA-DLPC films are squeezed. These PSMA-DLPC complexes also exhibit a more distinct liquid expanded phase, which indicates they are more resistant to compression, suggesting a varied self assembly, at the air-water interface, in comparison to the behaviour of PSMA-DLPC complexes prepared from using lower molecular weight hydrophilic PSMA-based lipid solubilising agents.

Figure 6.31, shows the molecular orientation of three lipid solubilising agents, functional groups, at the oil-water interface. Figure 6.31 illustrates that for the hydrophobic materials, there is more hydrophobic region for the hydrophobic phospholipids to interact with.

System A, which represents the ALT-PSMA triad sequence, this will be more abundant in the lower molecular hydrophilic PSMA-based copolymers (1:1 molar

## **-Chapter 6-**

ratio) - NR1 solution. For this system, the single styrene monomer units will be available in smaller areas, for the hydrophobic phospholipids to interact with.

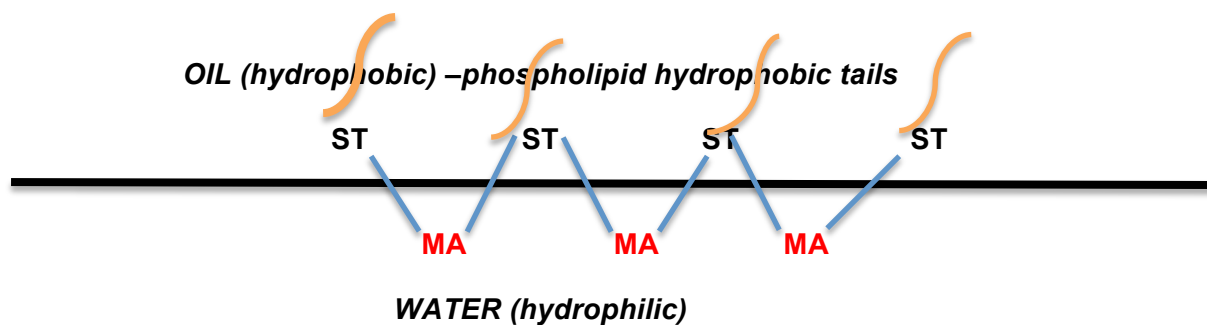
In comparison to system B, which is most prevalent in the MW 9, 500, blocky 3:1; ST: MA monomer feed ratio, PSMA-based copolymer (NR3), for the NON-ALT triad sequence regions, here there is higher concentration and a larger hydrophobic styrene area for the phospholipid hydrophobic tails to interact with. Perhaps these polymer regions will wrap the phospholipid bilayer tighter and more effectively. This explains why a better surface activity is attained for the complexes that are formulated with hydrolysed PSMA-based copolymer NR3.

System C shows the triad sequence that is most likely to be present for the PSMA, partial methyl ester (10-15%), MW 350, 000, 1:1 molar ratio, lipid solubilising agent. This SEMI-ALT triad monomer sequence will not only have single hydrophobic styrene monomers, but this effective lipid solubilising agent possesses additional hydrophobic ester tails that are also able to orientate towards the hydrophobic phospholipid tails, at the oil-water interface. This justifies why the Langmuir features for the PSMA-phospholipid complexes that are generated from this lipid solubilising agent exhibits unique features, as varied self-assembly is evidently present.

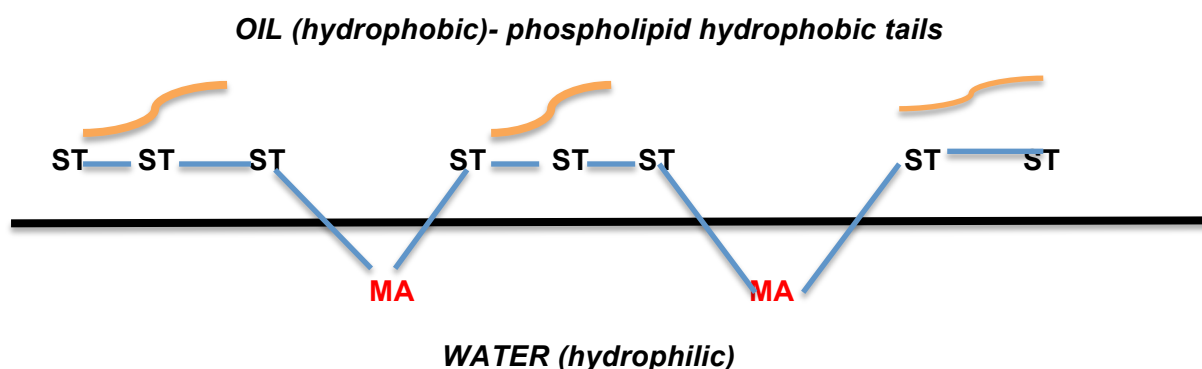
In conclusion, the surface behaviour of the polymer phospholipid complexes generated can be optimised by carefully selecting the lipid solubilising agent and NR6 and NR3 seem to be the ideal materials for selection based on the experimental data obtained.

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(A) ALT – PSMA REGION, AT OIL-WATER INTERFACE - NR1



(B) NON-ALT, PSMA REGION, AT OIL-WATER INTERFACE – NR



(C) SEMI-ALT (partial ester) PSMA REGION, AT OIL-WATER INTERFACE - NR6

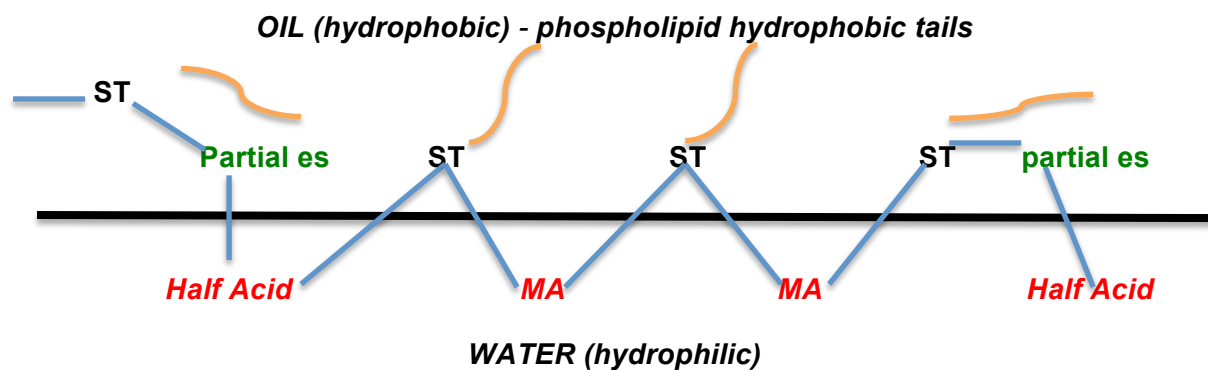


Figure 6.31. Molecular orientation of maleic acid, styrene, partial ester functional groups, at the oil-water interface, for (A) ALT(B) NON-ALT and (C) SEMI-ALT (partial ester) PSMA-based copolymers towards hydrophobic phospholipid tail.

**CHAPTER 7:  
THE ROLE AND LIMITATIONS OF  
HYALURONAN AS AN ORTHOPEADIC  
AND OPHTHALMIC BIOLUBRICANT;  
STRUCTURE-PROPERTY  
RELATIONSHIPS**

## **7.0 Introduction**

The employment of HA in biolubrication applications is economically feasible now, as it is readily available from bacterial fermentation processes, in a thermally stable form, known as HyaCare® [126]. Despite this advancement in HA research, section 7.1 emphasises that the source of HA will affect its performance. The commercial orthopaedic and ophthalmic HA products are evaluated in section 7.2. The introduction of HA into a contact lens and multipurpose solution (Safe-Gel™) is also investigated in section 7.3. The majority of this study is concerned with the use of HA in ophthalmic applications, where its attention and demand has recently increased. Finally, section 7.4 with reference to the *in-vitro* experiment data obtained, addresses the limitations of using HA alone in orthopaedic and ophthalmic biolubrication applications.

## **7.1 The study of HA solutions from different biological sources**

### **7.1.1 <sup>1</sup>H-NMR analysis of HA solutions**

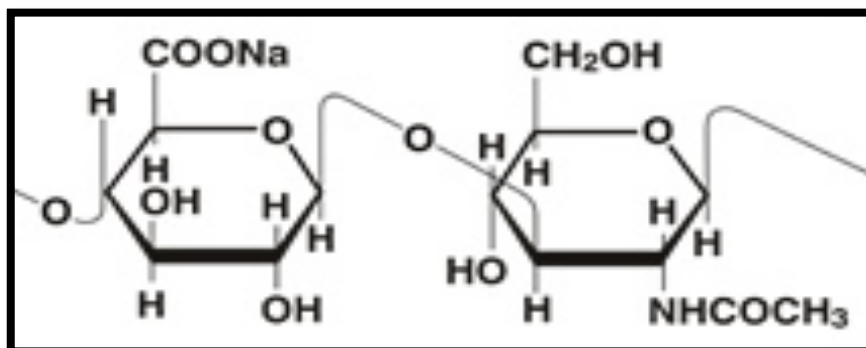
Nuclear magnetic resonance (NMR) is a physical phenomenon based upon the magnetic property of an atom's nucleus, the most often-used nuclei being hydrogen-1 and carbon-13. NMR spectroscopy is one of the principal techniques used to obtain physical, chemical, electronic and structural information about a molecule. NMR was the spectroscopic technique of choice since <sup>1</sup>H-NMR enables the study of hydrogen nuclei in molecules. <sup>1</sup>H-NMR spectroscopy was carried out, to deduce the structure of HA and furthermore to determine whether or not the copolymer composition of HA was source dependent or not.

Table 7.1. Biological sources of HA characterised by <sup>1</sup>H-NMR spectroscopy.

<b><i>Biological HA source characterised</i></b>	<b><i>Supplier</i></b>	<b><i>Solvent</i></b>
bovine vitreous humour	Sigma-Aldrich	D <sub>2</sub> O
rooster comb	Sigma-Aldrich	D <sub>2</sub> O
bacterial fermentation ( <i>Streptococcus Zooepidemicus</i> )	Sigma-Aldrich	D <sub>2</sub> O
human umbilical cord	Sigma-Aldrich	D <sub>2</sub> O

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HA from different biological sources, were dissolved into deuterated water ( $D_2O$ ). 32 scans were collected for each sample, on a 300MHz NMR spectrometer. The Fourier transform was processed using the XWIN-NMR 3.5 Bruker NMR software.




1,  $-\underline{CH}-$ , 2,  $-\underline{OH}-$ , 3,  $-\underline{COOH}-$ , 4,  $-\underline{NHCOCH}_3-$ , 5,  $-\underline{CH}_2OH-$ , 6,  $-\underline{NHCOCH}_3-$

Figure 7.1. Types of hydrogen chemical environments for HA repeat unit.

As deuterium oxide  $D_2O$  was used as the solvent for the NMR analysis,  $D_2O$  exchanges with liable protons such as  $-ROH-$ ,  $-RCOOH-$  and  $-RNH-$ . Due to the rapidity of the exchange, ROH becomes ROD, RCOOH becomes RCOOD and RNH becomes RND.



So peaks observed for the liable protons disappear (or are diminished) and a peak corresponding to  $H \cdots O \cdots D$  appears around 5  (ppm). In this work the HOD peak was identified at 4.6ppm. In theory, we should only see four different main chemical environments for the HA solution (in this deuterated solvent system), as can be identified and labelled in Figure 7.2 and Table 7.2.

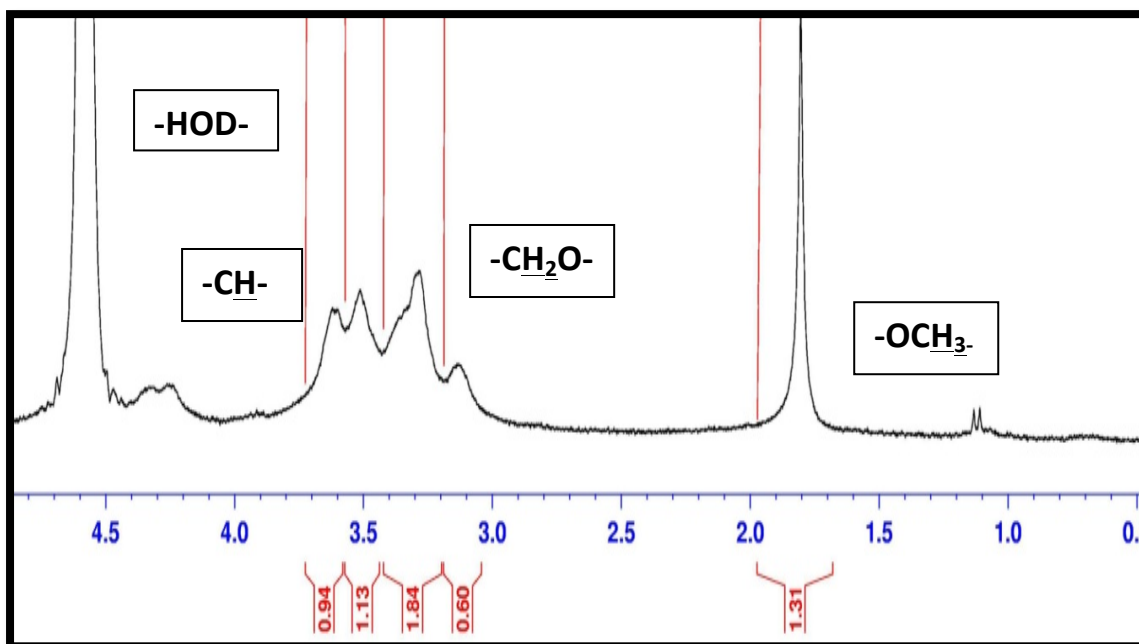


Figure 7.2.  $^1\text{H}$ -NMR spectra of HA from *Streptococcus Zooepidemicus* in  $\text{D}_2\text{O}$  (at room temperature) using a 300 MHz NMR spectrometer.

Table 7.2. Chemical shift assignments for  $^1\text{H}$ -NMR spectra of HA from *Streptococcus Zooepidemicus*.

<b>Chemical shift <math>\delta</math> (ppm)</b>	<b>Functional group</b>	<b>Monomer Component</b>	<b>Position (see Figure 7.1)</b>
1.8	-OCH <sub>3</sub> -	acetyl glucosamine	6
3.1	-CH <sub>2</sub> O-	acetyl glucosamine	5
3.3	-CH <sub>2</sub> O-	acetyl glucosamine	5
3.4	-CH-	both	1
3.5	-CH-	both	1
3.6	-CH-	both	1
4.2 - 4.4	-CH-	both	1
4.6	-HOD-peak	due to $\text{D}_2\text{O}$ exchange	

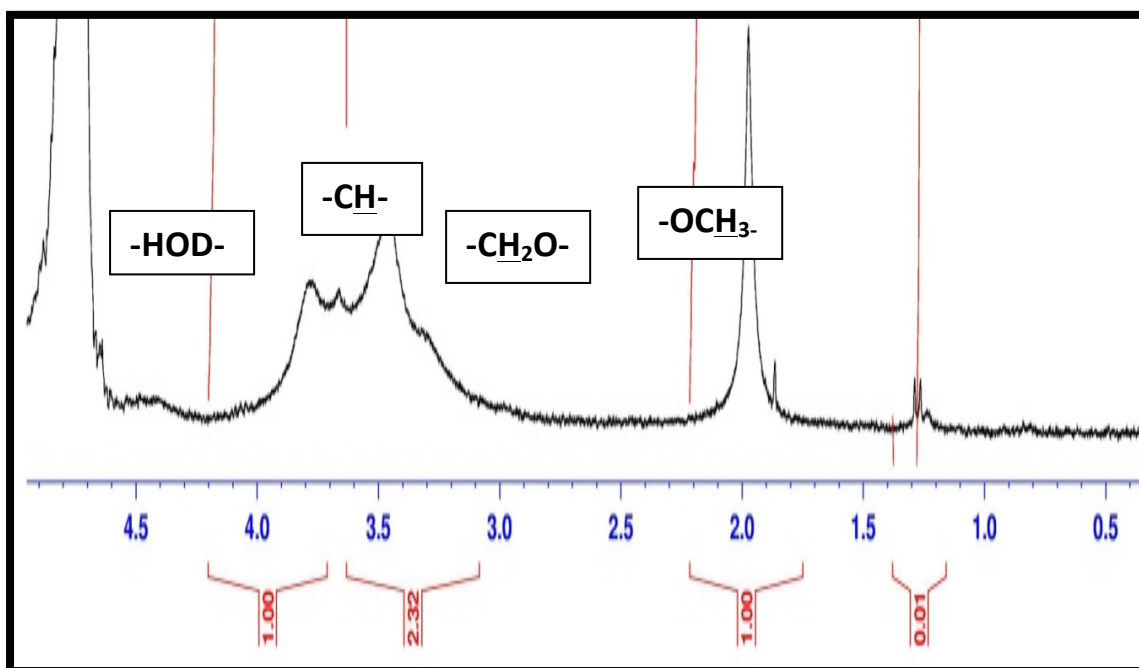


Figure 7.3.  $^1\text{H}$ -NMR spectra of HA from human umbilical cord in  $\text{D}_2\text{O}$  (at room temperature) using a 300  $\text{MHz}$  NMR spectrometer.

Table 7.3. Chemical shift assignments for  $^1\text{H}$ -NMR spectra of HA from human umbilical cord.

Chemical shift (ppm)	Functional group	Monomer component	Position (see Figure 7.1)
2.0	$-\text{OCH}_3-$	acetyl glucosamine	6
3.1	$-\text{CH}_2\text{O}-$	acetyl glucosamine	5
3.3	$-\text{CH}_2\text{O}-$	acetyl glucosamine	5
3.4	$-\text{CH}-$	both	1
3.5-3.8	$-\text{CH}-$	both	1
4.6	$-\text{HOD}-$ peak	due to $\text{D}_2\text{O}$ exchange	1



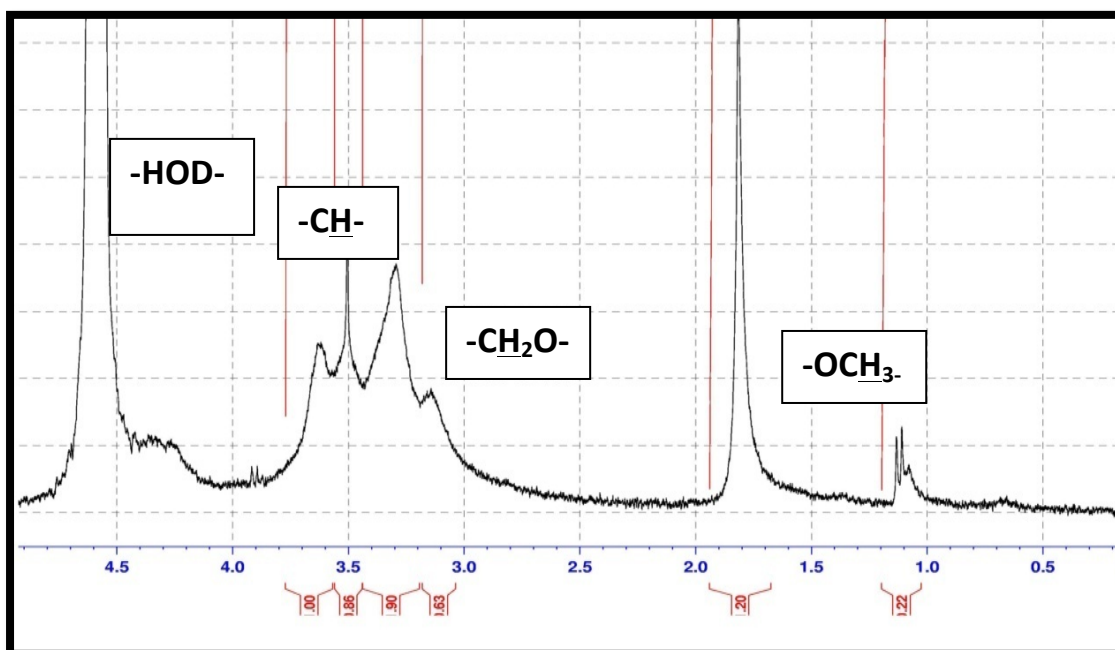


Figure 7.4  $^1\text{H}$ -NMR of HA from bovine vitreous humour in  $\text{D}_2\text{O}$  (at room temperature) using a 300  $\text{MHz}$  NMR spectrometer.

Table 7.4. Chemical shift assignments for  $^1\text{H}$ -NMR spectra of HA from bovine vitreous humour.

<b>Chemical shift (ppm)</b>	<b>Functional group</b>	<b>Monomer component</b>	<b>Position (see Figure 7.1)</b>
1.8	$-\text{OCH}_3-$	acetyl glucosamine	6
3.1	$-\text{CH}_2\text{O}-$	acetyl glucosamine	5
3.3	$-\text{CH}_2\text{O}-$	acetyl glucosamine	5
3.4	$-\text{CH}-$	both	1
3.5	$-\text{CH}-$	both	1
3.6	$-\text{CH}-$	both	1
4.6	$-\text{HOD}-$ peak	due to $\text{D}_2\text{O}$ exchange	

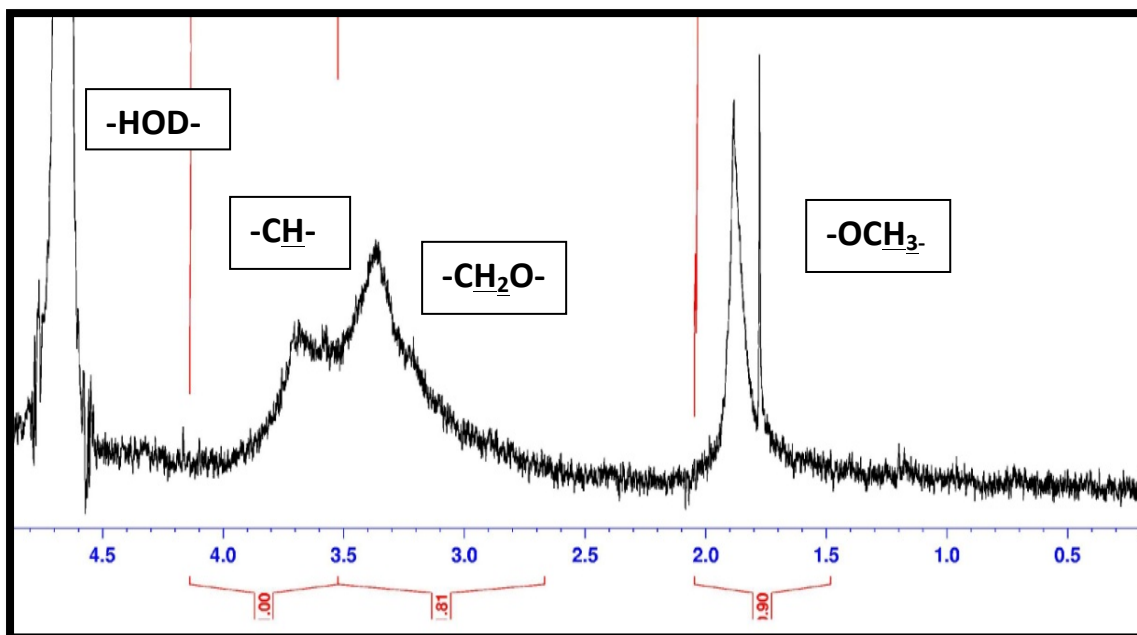


Figure 7.5  $^1\text{H}$ -NMR spectra of HA from rooster comb in  $\text{D}_2\text{O}$  at room temperature using a 300 MHz NMR spectrometer

Table 7.5. Chemical shift assignments for  $^1\text{H}$ -NMR spectra of HA from rooster comb.

<b>Chemical shift <math>\delta</math> (ppm)</b>	<b>Functional group</b>	<b>Monomer component</b>	<b>Position (see Figure 7.1)</b>
1.8	-OCH <sub>3</sub> -	acetyl glucosamine	6
3.2	-CH <sub>2</sub> O -	acetyl glucosamine	5
3.3	-CH <sub>2</sub> O -	acetyl glucosamine	5
3.4	-CH-	both	1
3.5	-CH-	both	1
3.7	-CH-	both	1
4.6	-HOD- peak	due to $\text{D}_2\text{O}$ exchange	

For each HA biological source studied, three main hydrogen chemical environments can be identified from the  $^1\text{H}$ -NMR spectra attained. Two are due to the acetyl glucosamine monomer unit (-OCH<sub>3</sub> - 2ppm, -CH<sub>2</sub>O - 3-3.5ppm) and one due to CH- (3.4ppm-3.7ppm, 4.8ppm) for both monomer units, respectively. See Figure 7.1 for the expected hydrogen chemical environments for the HA polymers, no quantitative analysis was conducted as this was not required for the purpose of this study. In order to investigate the effects of increasing temperature on improving the resolution, a HA solution (bovine vitreous humour sourced) was heated at 60<sup>0</sup>C in the NMR spectrometer and the  $^1\text{H}$ -NMR was collected (on a 300MHz NMR spectrometer). The Fourier transform was processed using the XWIN-NMR 3.5 Bruker NMR software. The solvent employed was kept the same ( $\text{D}_2\text{O}$ ). 32 scans were collected and the spectrum is presented in Figure 7.6. What is apparent is that increasing the temperature, improved the resolution and peak assignments were much easier to clarify, as peaks appeared much sharper, increased temperature reduces

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intermolecular hydrogen bonding, so the resonance positions for these protons are temperature dependent. As the temperature is changed, the populations of conformations are altered and the observed average chemical shift value may change. The changes in chemical shifts at different temperatures are often enough to resolve resonance which may have overlapped with one another at room temperature. When the temperature is changed, the populations of available conformations change and the degree of intra-molecular hydrogen bonding is affected with dramatic changes in the chemical shifts of the -OH resonances. At higher temperatures the correlation time is shorter and at lower temperatures the correlation time is longer. Therefore at as the temperature is decreased one expects the correlation time to increase and the  $T_2$  to decrease. Since the line width at half height is  $1/(\pi \cdot T_2)$ , the line width increases as temperature is decreased.

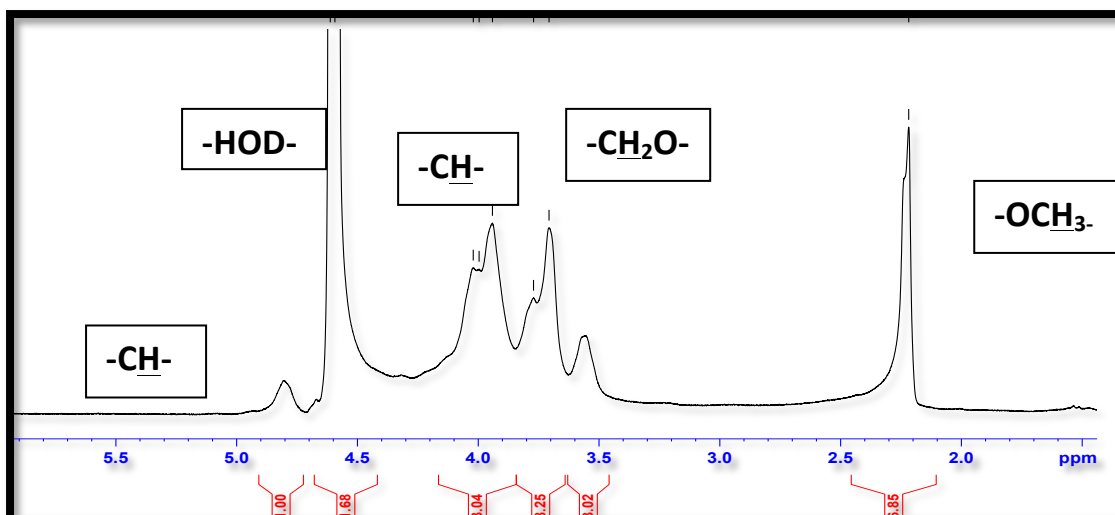


Figure 7.6.  $^1\text{H}$ -NMR spectra of HA from bovine vitreous humour of the eye in  $\text{D}_2\text{O}$  at  $60^\circ\text{C}$  using a 300  $\text{MHz}$  NMR spectrometer.

Table 7.6 Chemical shifts assignments for functional groups in bovine vitreous humour HA

Chemical shift (ppm)	Functional group	Monomer component	Position (see Figure 7.1)
2.3	-OCH <sub>3</sub> -	acetyl glucosamine	6
3.5	-CH <sub>2</sub> O -	acetyl glucosamine	5
3.7	-CH <sub>2</sub> O -	acetyl glucosamine	5
3.8	-CH-	both	1
4.0	-CH	both	1
4.1	-CH-	both	1
4.6	-HOD- peak	due to $\text{D}_2\text{O}$ exchange	1
4.8	-CH-	both	

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HA can be isolated from several sources (e.g., bacterial, rooster comb, umbilical cord and vitreous humour, etc.) and therefore can possess diverse impurities.  $^1\text{H}$ -NMR analysis revealed that the copolymer composition and purity varied with the biological source it was extracted from (see Figures 7.2-7.6 for spectra obtained). Qualitative  $^1\text{H}$ -NMR analysis illustrates that the peaks for each biological sourced HA are not identical. This provides evidence that the purity, copolymer composition and perhaps the molecular weight are all source dependent. However, further NMR studies are necessary, for more detailed structural analysis to support these findings.

Aviva Shiedlin *et al* [127] measured impurities and the differences in biological activity between HA preparations from different sources. It was demonstrated that nucleic acid and protein content was highest in human umbilical cord and bovine vitreous HA, and was low in bacterial and rooster comb HA. Macrophages exposed to human umbilical cord HA produced significantly higher amounts of TNF- $\alpha$  relative to control or bacterial-derived HA. These results indicate that the source of HA should be carefully selected, due to differences in features and types of contaminants that could be present, as a result of the HA isolation source, leading to widely different behaviours *in-vitro* and *in-vivo* [127-130]. HA copolymer composition seems to be source dependent, affecting the performance of HA, as will be revealed in the upcoming sections in this study.

### **7.1.2 Rheological and frictional properties**

HA occupies a large hydrated volume and therefore shows solute-solute interactions, at low concentrations. HA solutions show excluded volume effects, as it restricts access to other macromolecules. HA is a polyelectrolyte and therefore the HA solution properties were also greatly affected by ionic strength. Experimental data supports the view that intramolecular hydrogen bonds and polyanionic properties of HA both contribute and provide a highly expanded macromolecular conformation. The most plausible explanation for the large hydrodynamic volume of HA and hence other non-Newtonian viscoelastic properties is the presence of multiple dynamic hydrogen bonds between adjacent saccharides. This restricts rotation and flexion at the glycosidic bonds and creates a stiffened yet mobile polymer chain. The flexibility and permeability properties of the HA network can then be accounted for in terms of inter-chain hydrodynamic interactions of this extended structure, with entanglements being especially important at elevated concentrations. In a narrow range of acid pH

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around pH 2.5, HA does have different properties and these may reflect chain-chain association. In contrast, HA properties in the broad pH range 3-8 are incompatible with any significant intermolecular self-association that forms stable tertiary structures. At physiological pH, the properties of HA solutions can be predicted directly from the behaviour in dilute solutions and even at very high concentrations, individual chains remain mobile and they undergo no transition to a gel-like state. In dilute solution HA behaves as a stiffened random coil [131-134]. A few drops of the HA solution were placed onto the lower plate of a Bohlin CVO50 rheometer and the viscosity was measured as a function of shear stress using a CP1<sup>o</sup>/20mm upper plate at 34<sup>o</sup>C. See Chapter 3 (section 3.3) for the experimental set up, background theory and further experimental details. Figure 7.7 shows that 0.4%(w/v) of HA from rooster comb, *streptococcus zooepidemicus*, bovine vitreous humour and human umbilical cord exhibit shear thinning behaviour. The solutions at a higher concentration show non-Newtonian behaviour, with rooster comb sourced HA, being the most viscoelastic and therefore of the highest molecular weight. In concentrated solutions stiffened random coils of HA show chain entanglement and form viscoelastic solutions. 0.4%(w/v) solutions of HA mimic the reported non-Newtonian, viscoelastic rheological behaviour of tears. As the shear rate was applied to the HA solution the viscosity decreased. The rooster comb sourced HA showed the most viscoelastic behaviour and the umbilical cord sourced HA the lowest coefficient of friction. This may be due to the varying molecular weights and branching extents, of the various HA copolymers.

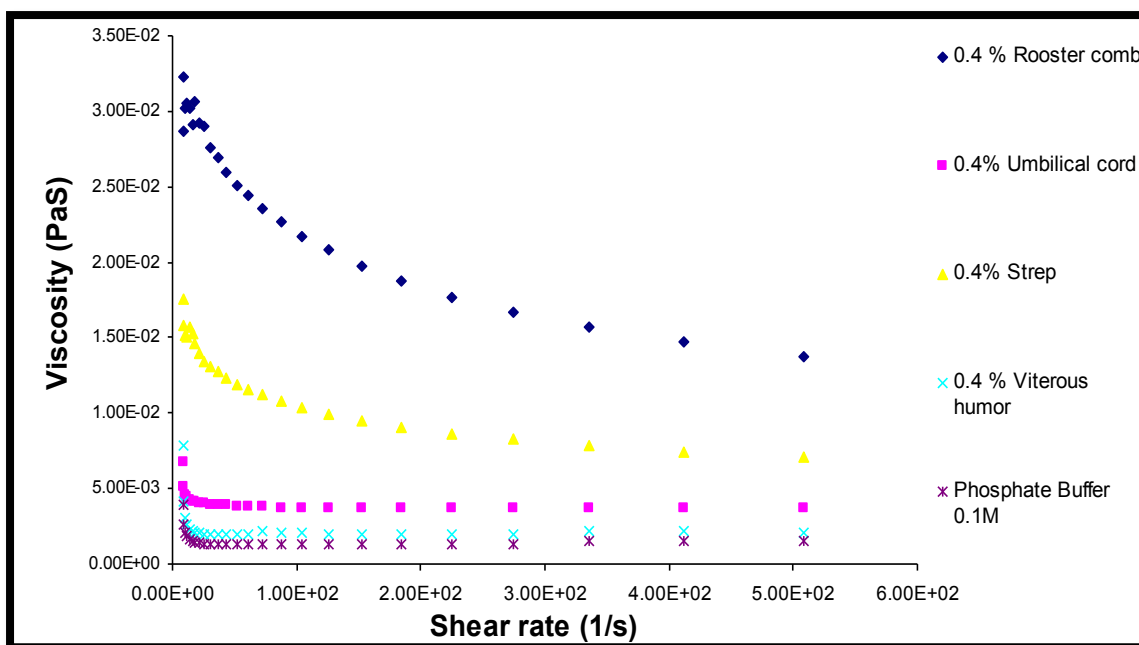


Figure 7.7. The viscosity of 0.4%(w/v) HA solutions in pH 7 phosphate buffer solution from different biological sources plotted against shear rate.

The frictional behaviour of 0.4%(w/v) HA solutions was examined using a CSM Nano Scratch Bio-tribometer, with a vibration free table. The frictional force of the sliding polypropylene head (attached to an etafilcon A contact lens, against the HA solution - 100 microlitres), which was placed onto a Melinex sheet (polyethylene terephthalate), was recorded as a function of distance travelled in mm (0-20mm). A load of 60mN (normal force) at a speed of 30mm/min was utilised. See section 3.4, Chapter 3 of this thesis for further experimental details. The only variable was the lubricant employed (0.1M phosphate buffer), which was used as a control for the investigation. The same experimental conditions were utilised for all frictional measurements in this study. The aim of this was to establish whether HA from different biological sources, possess varying lubricating capabilities.

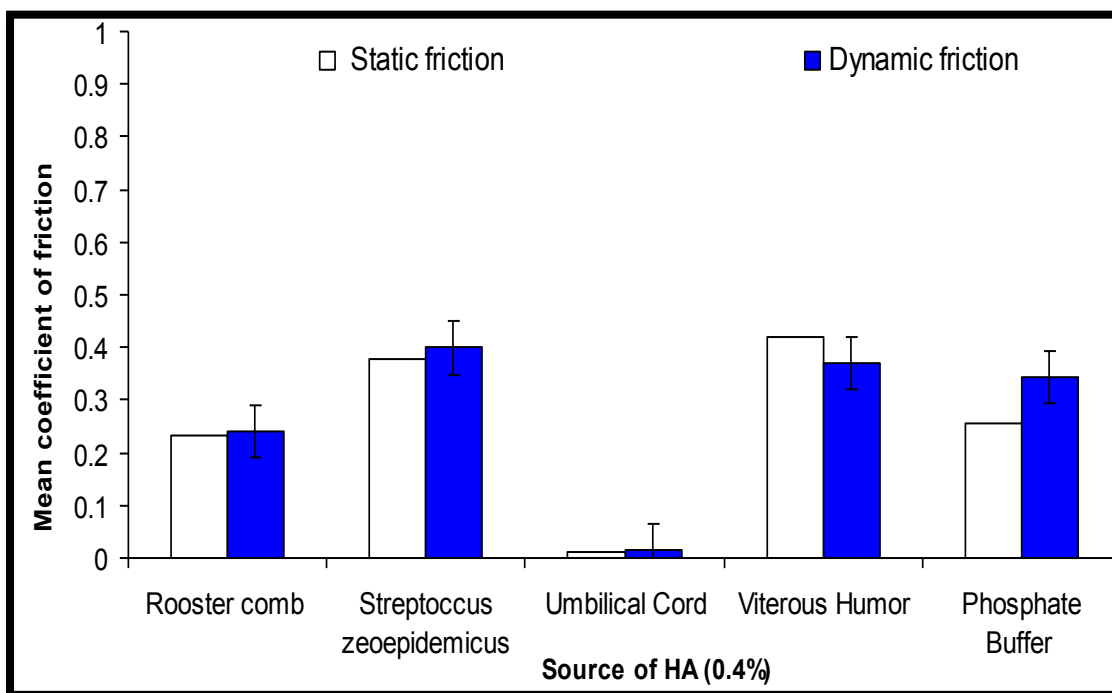


Figure 7.8. The frictional behaviour of 0.4%(w/v) HA in pH 7 phosphate buffer solution extracted from different biological sources with etafilcon A.

Figure 7.8 above, shows that the source of HA affects its lubricating capability. A 0.4 %(w/v) solution of umbilical cord HA exhibited the lowest coefficient of friction. This may be due the varying molecular weights of purity and degree of branching of the respected HA copolymer. Variations of the HA copolymer composition may also effect the lubricant capability of HA, because of its effects on water structuring around the HA chains, which in turn effects hydrogen bonding.

## 7.2 Evaluating HA commercial products

A few drops of Orthovisc was placed onto the lower plate of a Bohlin CVO50 rheometer and the viscosity measured as a function of shear stress, using a CP1<sup>o</sup>/20mm upper plate at 34<sup>o</sup>C. It was repeated at least three times in order to assess the reliability of the data obtained. Figure 7.9 and 7.10, demonstrate that HA viscosupplement injections (Orthovisc, in this case) are extremely viscoelastic and resist shear forces. Figure 6.10 also shows that Orthovisc exhibits Newtonian rheological behaviour, and does not mimic the rheological behaviour of native synovial fluid. As the shear rate was increased the viscosity of the HA solution remained the same. Nevertheless, Orthovisc also did not undergo any detectable chain scission as a consequence of mechanical degradation, due to the shear forces applied to Orthovisc, via the mechanical action of the rotating cone and plate. Chain

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scission would have been observed if the viscosity reduced as the shear rate increased, indicating a decrease in molecular weight. Figure 7.11, depicts that Orthovisc has a low coefficient of friction ( $\sim 0.09$ ). This demonstrates that HA is a more effective lubricant at higher concentration and in a purer form.  $^1\text{H}$ -NMR analysis of the highly viscous Orthovisc solution did not disclose any structural differences (see Figure 7.12). The differences in frictional and rheological behaviour are most likely due to concentration, which in turn is going to be related to molecular weight and more importantly due to the purity of HA in commercially available Orthovisc. It must also be noted that the HA chains in Orthovisc produced may be crosslinked.

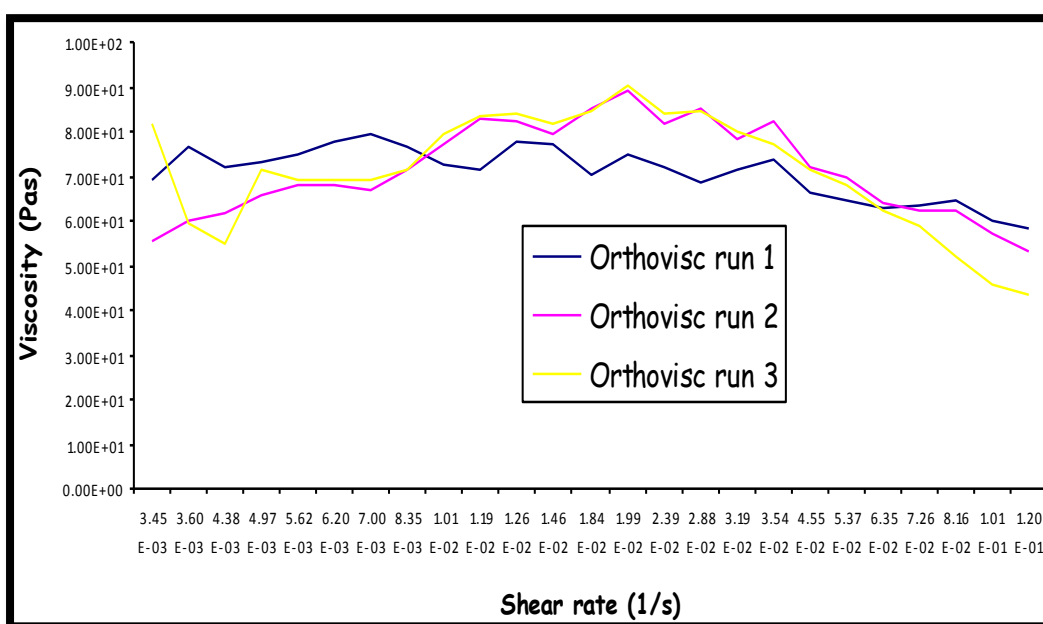


Figure 7.9. Rheological behaviour of Orthovisc, under mechanical shearing (same sample for each run)



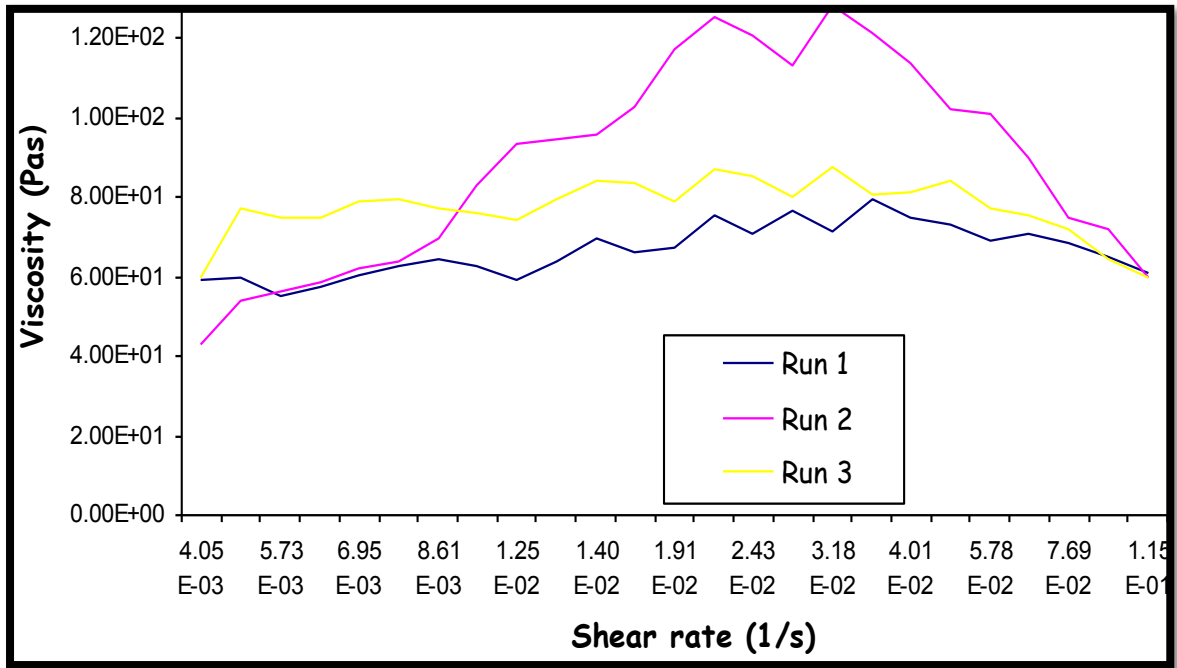


Figure 7.10. Rheological behaviour of Orthovisc, exhibiting Newtonian behaviour (fresh sample for each run).

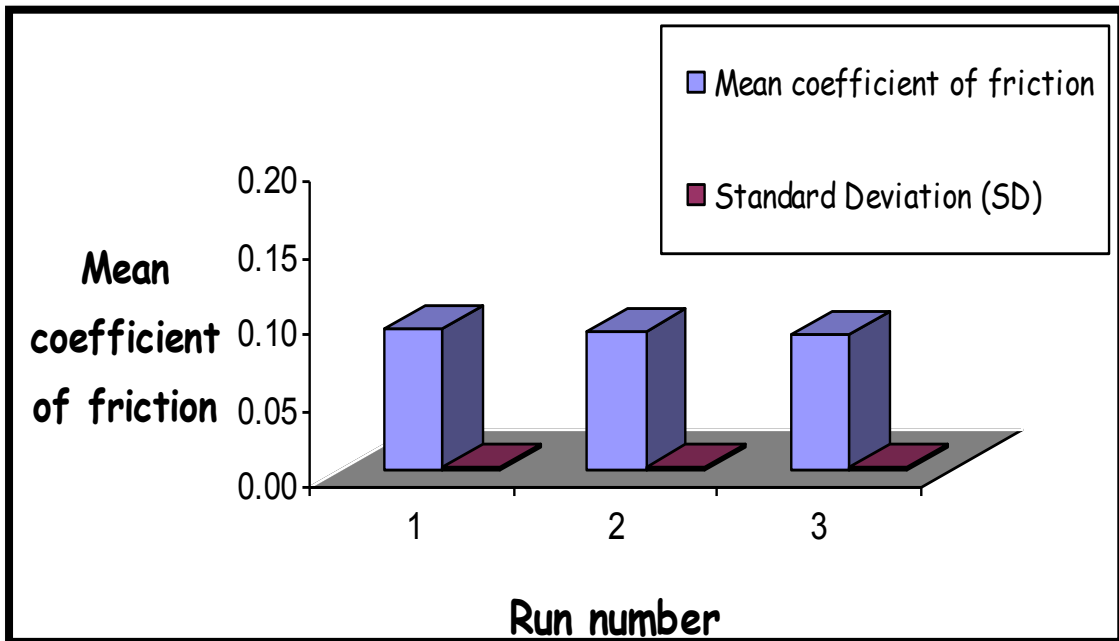


Figure 7.11. Frictional behaviour of Orthovisc, showing a very low mean coefficient of friction ( $\sim 0.09$ ).

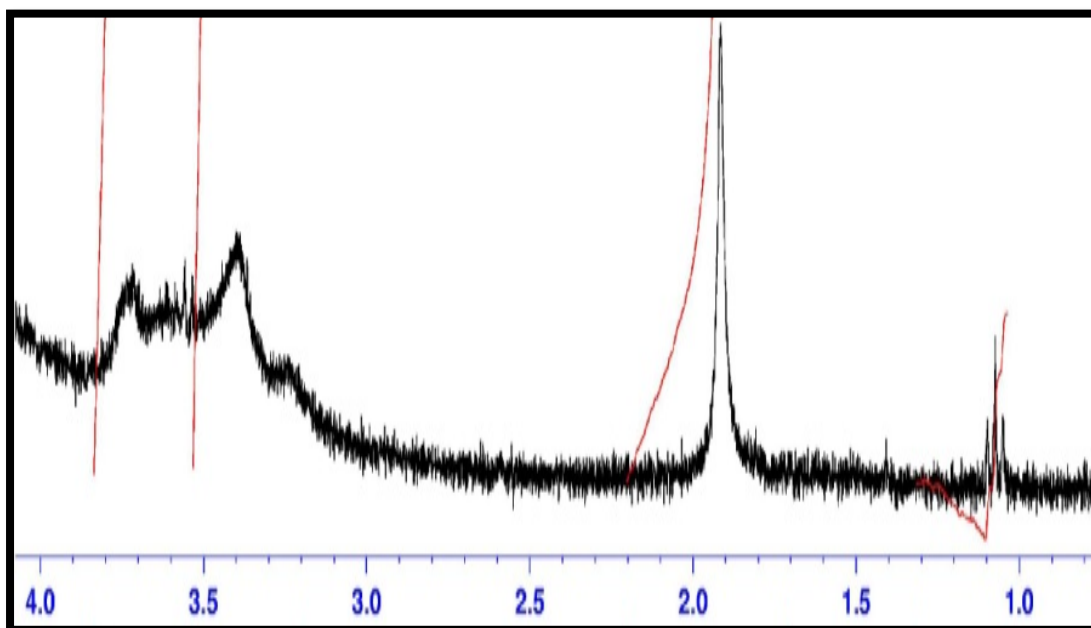


Figure 7.12. <sup>1</sup>H-NMR spectra of HA from Orthovisc.

The most important reason why HA is employed in artificial tears is that it is biocompatible, as it is a natural biopolymer that is already present in the ocular environment. It is believed to provide excellent lubrication and hydration. At low shear rates (when the eye is open), HA molecules are random and entangled, so the viscosity of the HA solution is high. At higher shear rates (during blinking), the molecules separate and elongate, resulting in a greatly decreased viscosity of the solution, which can easily be spread over the ocular surface. It is a superior viscous ingredient for ophthalmic solutions. Additionally, sodium hyaluronate is presently the most common mucoregulator/mucomimetic (see Table 7.7).

HA artificial tears are dilute HA solutions, largely bacterial fermentation sourced. The attractive choice of HA into artificial tears is due to its ability to generate viscous solutions at low concentrations and its cellular healing capability reported by scientists over the years [135-150].

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Table 7.7. Some HA-based artificial tears [135-150].

\* = Osmolality

<b>Generic name</b>	<b>% HA(w/v)</b>	<b>Supplier</b>	<b>Source</b>
Blink™ Contacts	0.15	AMO (266 milliosmol)*	rooster comb
Vismed™	0.18	SD Health care	bacterial fermentation
Vizualize™ Dry Eye	0.1	Peach pharmaceuticals (294 milliosmol) *	bacterial fermentation
Hycosan™	0.1	Bausch & Lamb	
Hyalistil™	0.2	Sifi PHARMA	
Biolan™	0.15	Santen	bacterial fermentation
Oxyal™		Santen (273 milliosmol) *	bacterial fermentation
	0.15		
Hylashield™	0.15	Biomatrix	
Eyestil™	0.15	Lesi vision	
HyaCare®	0.1-0.4	Novozymes	bacterial fermentation
Ocumed®	0.1		
Hyal drop®	0.2	Fidia pharmaceutical s.p.a	functionalised HA (benzyl ester)
Focus Aquify™	0.1	Ciba Vision (296 milliosmol)*	bacterial fermentation
Avizor eye drops	0.1	Avizor	bacterial fermentation

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HA artificial tears are dilute solutions of HA and therefore do not appear very viscous. Moreover, they appear not to exhibit shear-thinning behaviour. The artificial tear with the highest concentration of HA (Blink™ Contacts) exhibited the most viscoelastic behaviour, as expected due to possessing a higher concentration of HA in solution (0.15%) as opposed to 0.1 % in Focus Aquify and Vizualize eye drops. The rheological behaviour of some HA-based artificial tears was measured on a Bohlin CV050 rheometer using a cone ( $1^0$ ) and plate (20 mm) at  $34^0\text{C}$ . Table 7.7, lists HA formulated artificial tears and indicates the generally low concentration of the HA component. As a consequence, these solutions do not show any distinct viscoelasticity or non-Newtonian behaviour (see Figure 7.13). Figure 7.7, discussed previously in this Chapter, also depicted that at least 0.4%(w/v) of HA is necessary in solution to exhibit non-Newtonian, shear thinning behaviour (pseudoplastic) that mimics that rheological behaviour of native tears. The employment of HA at such low concentration results in viscosity enhanced saline and not much more. The rooster comb source showed the most viscoelastic behaviour and the umbilical cord source the lowest coefficient of friction.

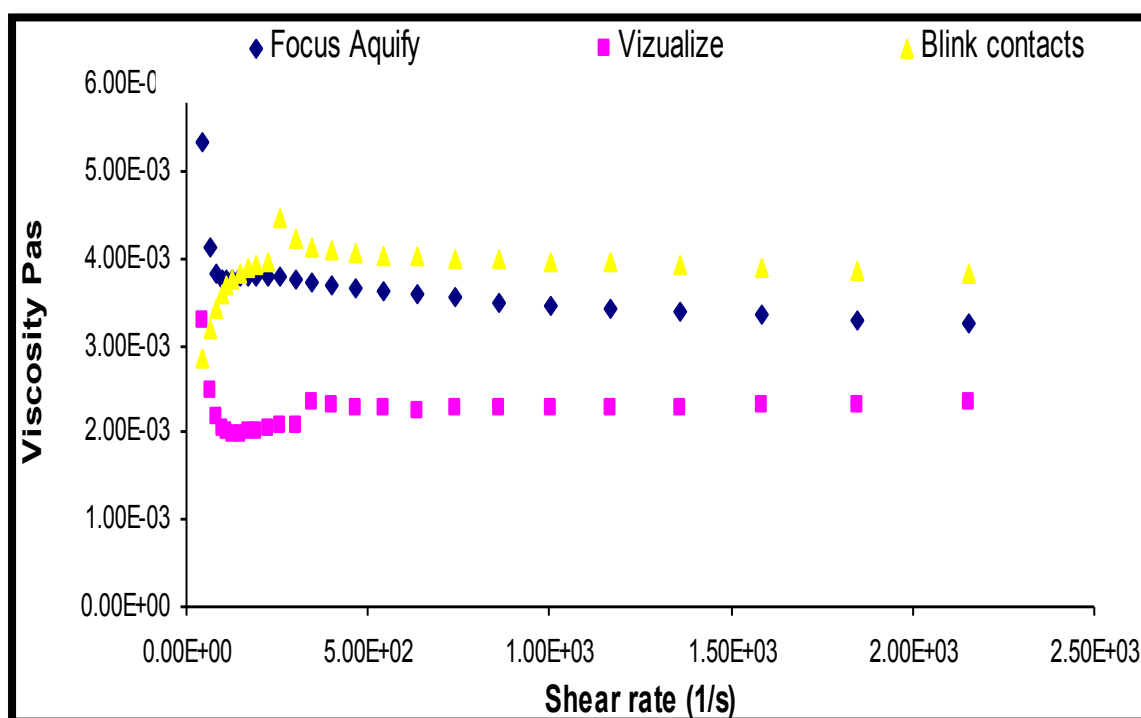


Figure 7.13. The viscosity of some HA-based artificial tears against shear rate.

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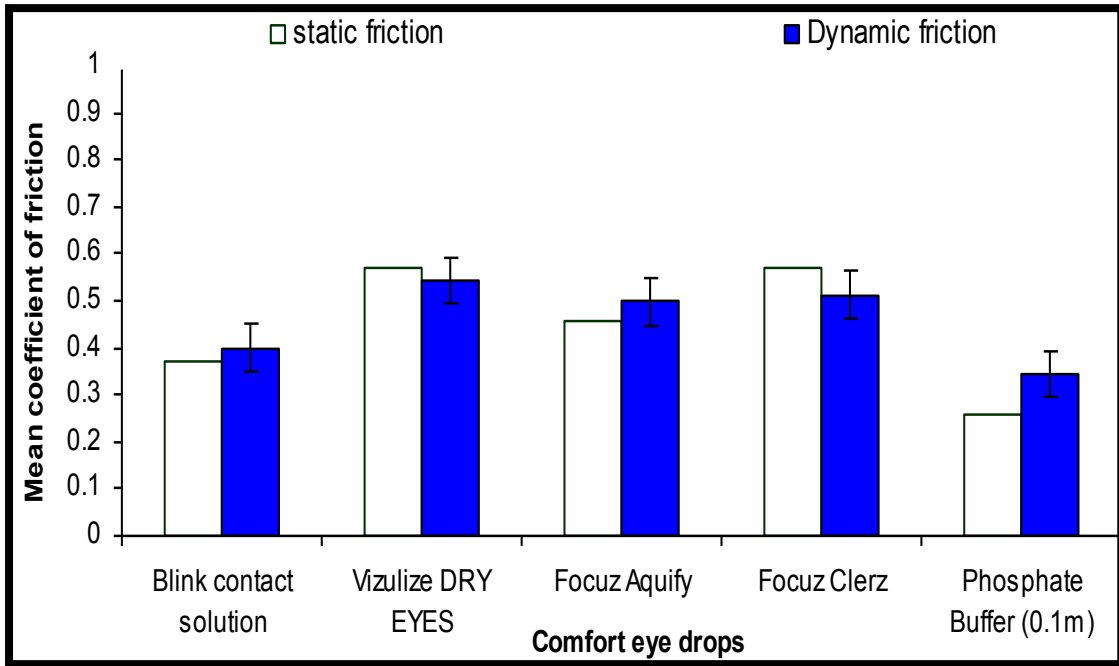


Figure 7.14. Frictional behaviour of HA-based artificial tears with etafilcon A.

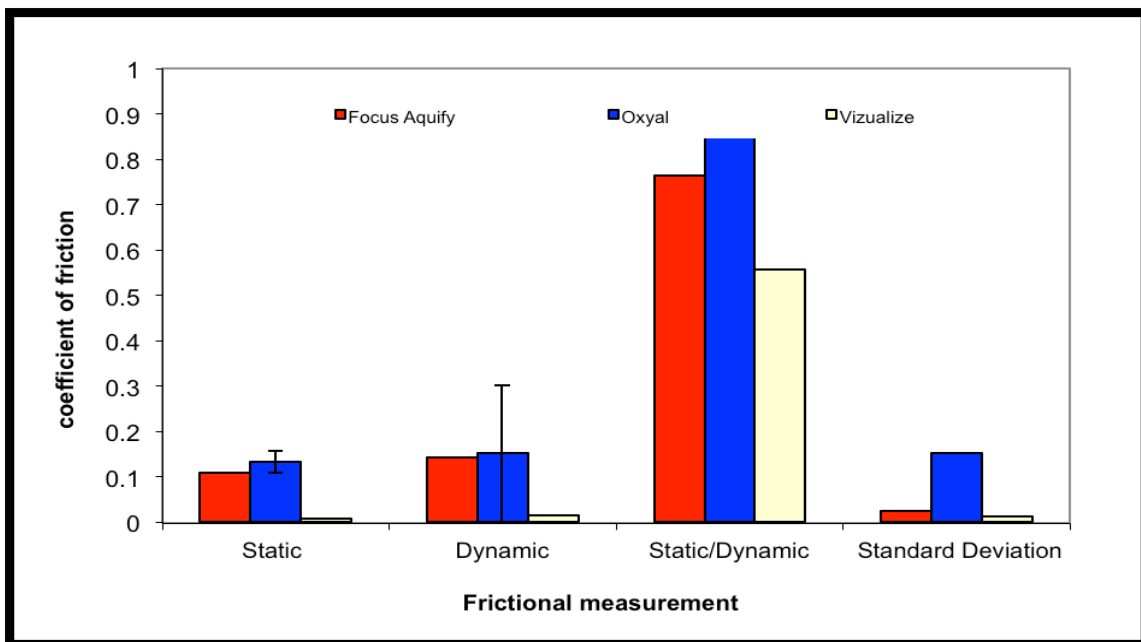


Figure 7.15. Frictional behaviour of HA-based artificial tears with nelfilcon A.

Figure 7.14 above, shows that generally HA-based artificial tears usually show a high coefficient of friction, although Blink™ Contacts, an artificial tear based from rooster comb, was lower than expected. Of all the HA-based artificial tears tested Blink® Contacts, 0.15% (w/v) HA, rooster comb sourced HA, exhibited the lowest osmolality, the most viscoelastic behaviour and the lowest coefficient of friction with etafilcon A contact lenses (see Figure 7.14).

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However, in contrast, HA-based artificial tears in combination with nelfilcon A (neutral flexible substrate) shows a far lower coefficient of friction than with etafilcon A (which is an ionic substrate), see Figure 7.15. This may be due to the fact that HA is negatively charged and is more compatible with a neutral surface, as opposed to an ionic surface like etafilcon A. Overall, HA-based artificial tears exhibited a higher frictional behaviour with etafilcon A ( $\geq 0.34$ ) than with nelfilcon A ( $\leq 0.15$ ).

The frictional behaviour of a contemporary HA-based artificial tear (Avizor moisture drops) was also carried with silicone hydrogel contact lens materials. Lower values were observed with galyfilcon A (0.15 - see Figure 7.16) than with lotrafilcon B (0.25 - see Figure 7.17). Overall, HA-based artificial tears in combination with silicone materials exhibit lower coefficients of friction. It is interesting to note that HA shows low frictional behaviour in combination with silicone hydrogels. The incorporation or association of HA into or with silicone hydrogels therefore warrants further study.

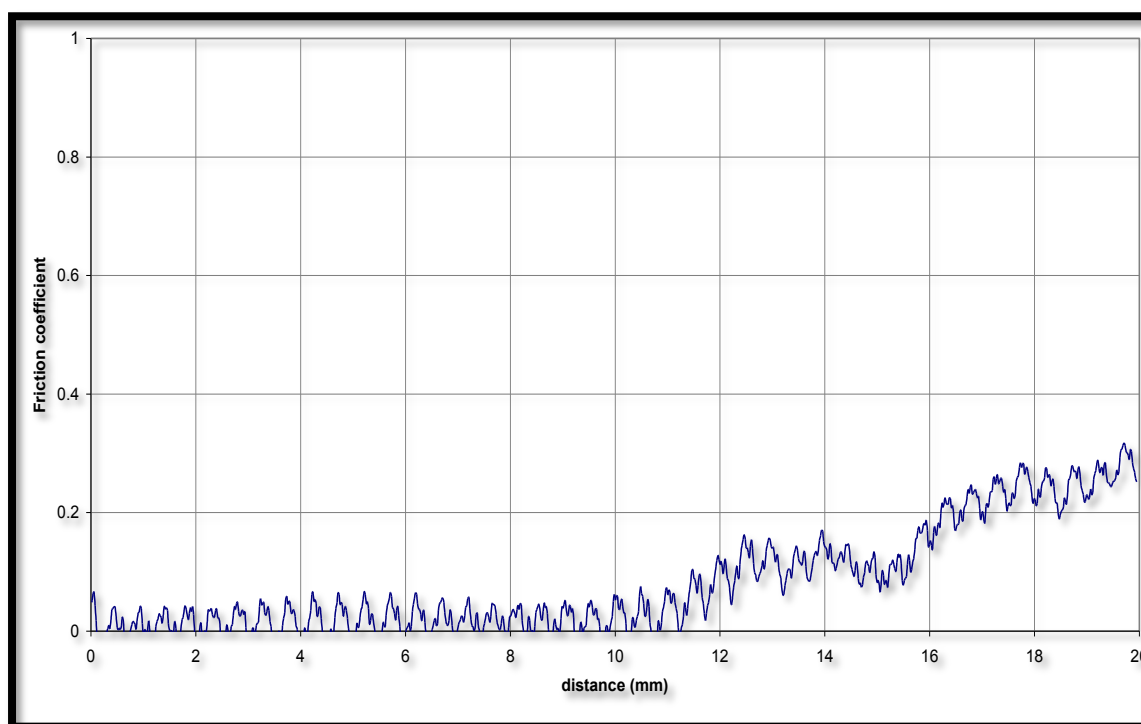


Figure 7.16. The coefficient of friction of galyfilcon A, against distance travelled with Avizor moisture drops, exhibiting an average coefficient of 0.15.

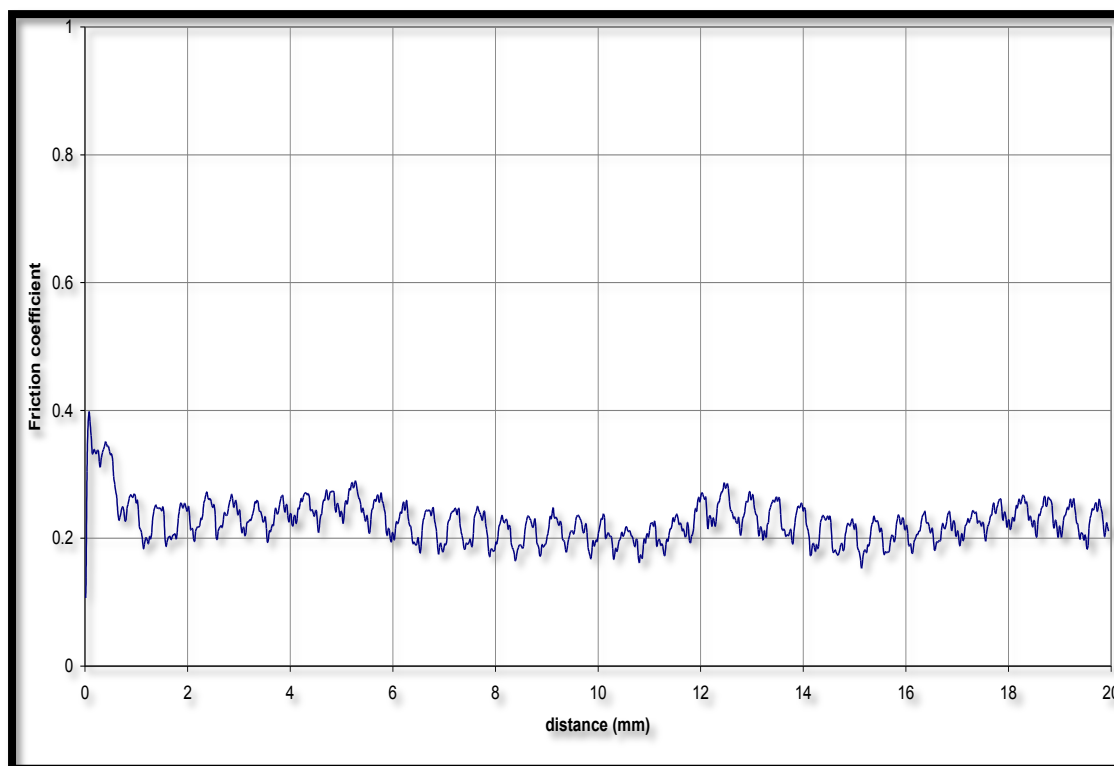


Figure 7.17. The coefficient of friction lotrafilcon B, against distance travelled with Avizor moisture drops, exhibiting an average coefficient of 0.25.

### 7.3 HyaCare®: A new class of HA

Over the years, the many different production technologies have been used to extract HA. When the ethically dubious method of extracting HA from rooster comb was replaced the more *Streptococcus* fermentation technology in 1990's, one might have believed that further progress in the production was impossible. Since then, Novozymes has developed a unique method for producing strain, *Bacillus Subtilis*. The method is based on Novozymes, advanced and safe fermentation and purification technology. *Bacillus Subtilis*, an "industry factory" since the 1960's, is a non-pathogenic organism that is easy to customise to meet various demands. *Bacillus Subtilis* is a safe organism producing GRAS (Generally Regarded as Safe) products. HyaCare® is a new HA - a breakthrough in consistency, safety and purity. It is available from Novozymes; Table 7.8, outlines some of its characteristics [126]. Table 7.8 lists the unique properties of HyaCare reported by Novozymes.

Table 7.8. Characteristics of HA in HyaCare® [126].

<b><i>HyaCare®</i></b>	<b><i>properties</i></b>
molecular weight	0.8MDa
sodium hyaluronate	94.6%
intrinsic viscosity	14.1 DL/G
pH of 0.5% aqueous solution	7.55
polydispersity Index	below 1.4

HyaCare® has greater water binding capability; Novozymes claim that 1 gram of HyaCare® binds up to 8 grams of water in infinite dilution. It exhibits greater water-binding capacity than high-molecular weight HA (1.5MDa). The unique process developed by Novozymes biopolymer is based on spray drying aqueous HA solutions and produces a very fine powder composed of micro and nanospheres. These particles have a very large surface area, which gives HyaCare® the best dissolving properties available in the market. HyaCare® is easier to filter and hence is more effectively purified. HyaCare® is also very stable towards heat sterilisation; the decrease in molecular weight of HyaCare® is only 10% after treated at 124°C for 8 minutes.

### **7.3.1 Safe-Gel™ : The first HA-based contact lenses [151-155].**

Optician (November 17, 2006) [156-157] reported the launch of a new lens in the UK, with a HA element, within the hydrogel matrix. The Safe-Gel™, is available as a 1-day and a 7-day and as a two weekly replacement lens. The introduction of HA into the Filcon B matrix, along with some other mineral components (magnesium, potassium and calcium) allows for its slow release as the lens reaches the ambient ocular temperature. The release is aimed to stabilise the tear film. Recent years has seen changes to lens materials so as to incorporate wetting agents within the lens matrix, thus changing the surface properties or releasing lubricants during wear. Safe-Gel™ was aimed to improve wearability in those patients with compromised tear films by incorporating HA gel within the biopolymer Filcon 1 material. The big challenge to researchers was to somehow incorporate the HA within the soft lens substance to allow its release with wear. This was possible due to HAs property of thermoreversibility. When HA is present in its gel state, there are many interchain links between the disaccharides components which are not true covalent bonds. Heating the solution to over 100°C results in the polymer chains becoming



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disorganised and passing into a normal liquid state. In this state it is possible to introduce the polymer evenly within the soft gel matrix and, when it is subsequently cooled, it recovers its organised state within the Filcon material. Another temperature increase, for example to the 'on eye' temperature of around 36°C, results in an increase in fluidity, so allowing a gradual release of the HA from the lens into the surrounding environment (the tears). This phenomenon is known as kinetic viscosity. This is now possible, especially with the ultra pure, thermally stable HA source - HyaCare®. The kinetic viscosity of HA decreases with increasing temperature and this decrease is more marked at lower temperatures than higher temperatures.. The dynamics of the 'flow' of HA into and out of a lens are therefore predictable knowing the local environment of the lens on the eye and exact formulation of the HA [151-157].



Scheme 7.1. The properties of Safe-Gel™ 1-day and Safe-Gel™ 7-day contact lens (HA-based contact lenses), reported by manufacturer. [151-157]

The 1-day material is a contact lens designed to be worn for one day and the 7-day material, for seven days respectively.

The frictional behaviour of Safe-Gel™ was conducted using a CSM Nano Scratch Bio-tribometer, with vibration free table allowing low coefficients of friction to be measured. The frictional force of the sliding polypropylene head (hydrophobic surface-attached to the Safe-Gel™), against the lubricant (HypoTears®, Safe-Gel™

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packing solution and Ciba Saline), which was placed onto the Melinex sheet (polyethylene terephthalate) under study, was recorded as a function of distance travelled in mm (0-20mm). A load of 60mN (normal force) at a speed of 30mm/min was utilised throughout. The only variable was the lubricant employed (100 microlitres). The static coefficient of friction was calculated as the average coefficient from 0-4mm of distance travelled, and the dynamic coefficient of friction from 4mm-20mm, respectively. The mean coefficient of friction was taken, from 0 to 20mm. Figure 7.18, illustrates that the frictional behaviour of Safe-Gel<sup>TM</sup> was dependent on the lubricant utilised. The lowest coefficient of friction was achieved in combination with HypoTear<sup>®</sup>, a PVA-PEG based artificial tear, preserved with benzalkonium chloride (BAK). When used with Safe-Gel<sup>TM</sup> packing solution (which consist of 0.1-0.3% (w/v) HA), a considerable increase in friction was observed. High levels of friction were observed with Ciba Saline (commercial saline from Ciba Vision).

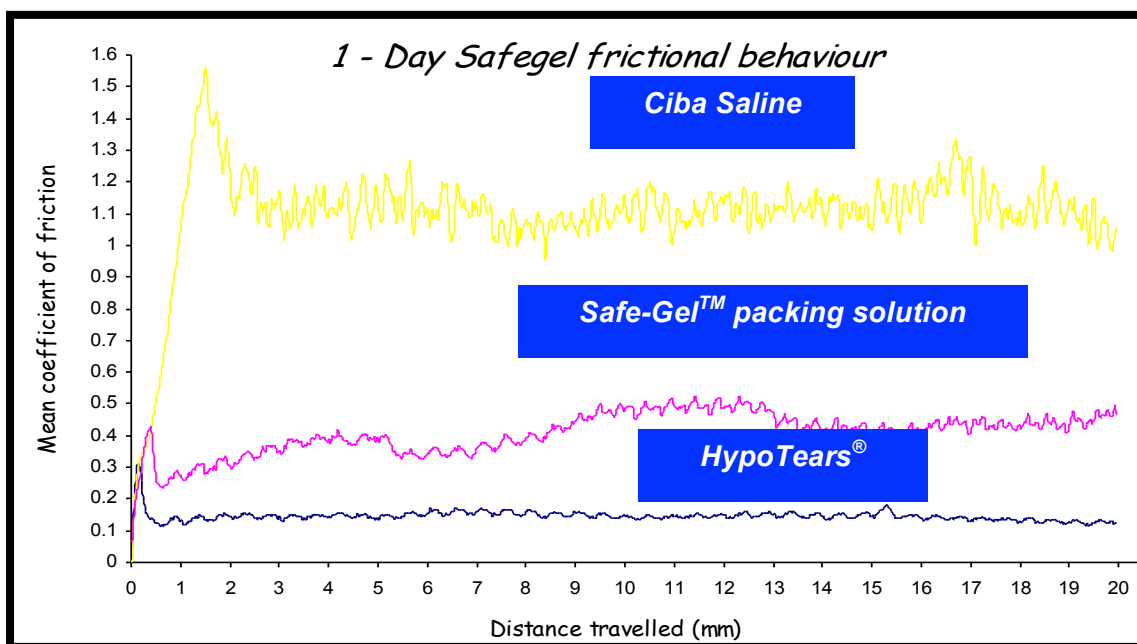


Figure 7.18. The mean coefficient of friction of 1-day Safe-Gel<sup>TM</sup> in combination with Ciba Saline, Safe-Gel<sup>TM</sup> packing solution and HypoTears<sup>®</sup>.

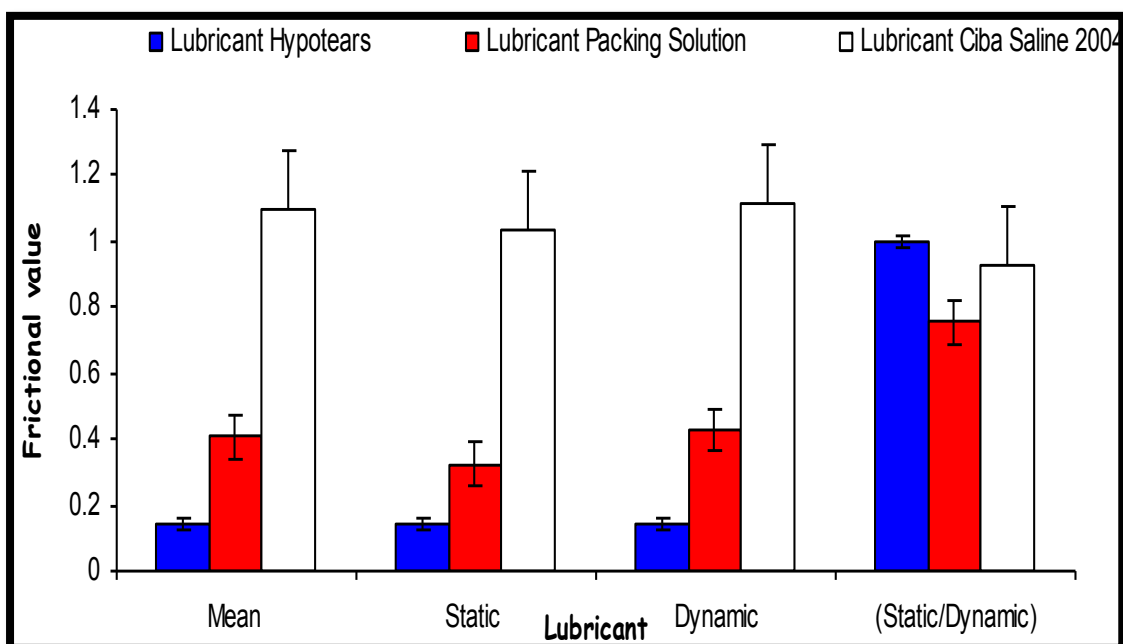


Figure 7.19. A summary of the frictional behaviour of 1-Day Safe-Gel™ contact lenses, with different lubricants.

Figure 7.20, depicts that 7-Day Safe-Gel™ showed a lower coefficient overall in comparison to 1-Day Safe-Gel™. To further understand these results the surface activity and the osmolality of the lubricants utilised were studied.

Measuring the osmolality (as described in Chapter 3 (section 3.5) for the lubricant utilised revealed that the 7-day Safe-Gel™ packing solution exhibited a higher osmolality than the 1-day Safe-Gel™ packing solution. This implies that perhaps that a higher concentration of HA is incorporated into the 7-Day Safe-Gel lens, or in the lens packing solution. This may help explain why lower levels of friction are seen with the 7 - Day Safe-Gel™ contact lenses.

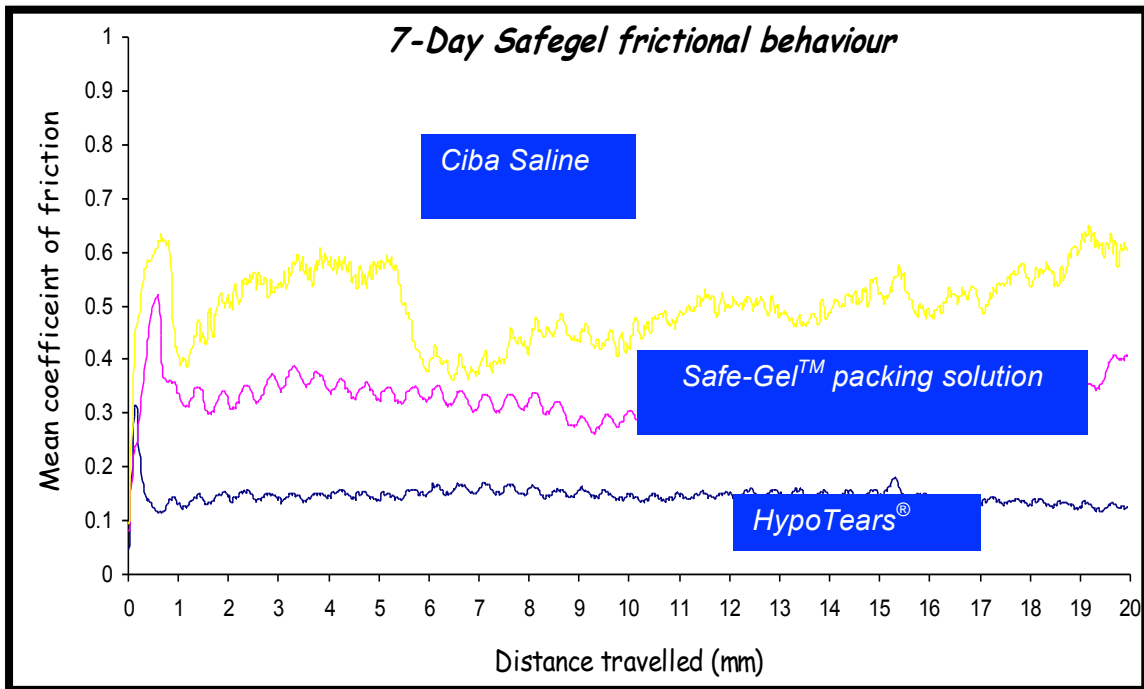


Figure 7.20. The mean coefficient of friction of 7-day Safe-Gel™ in combination with Ciba Saline, 7-day Safe-Gel™ packing solution and HypoTears®.

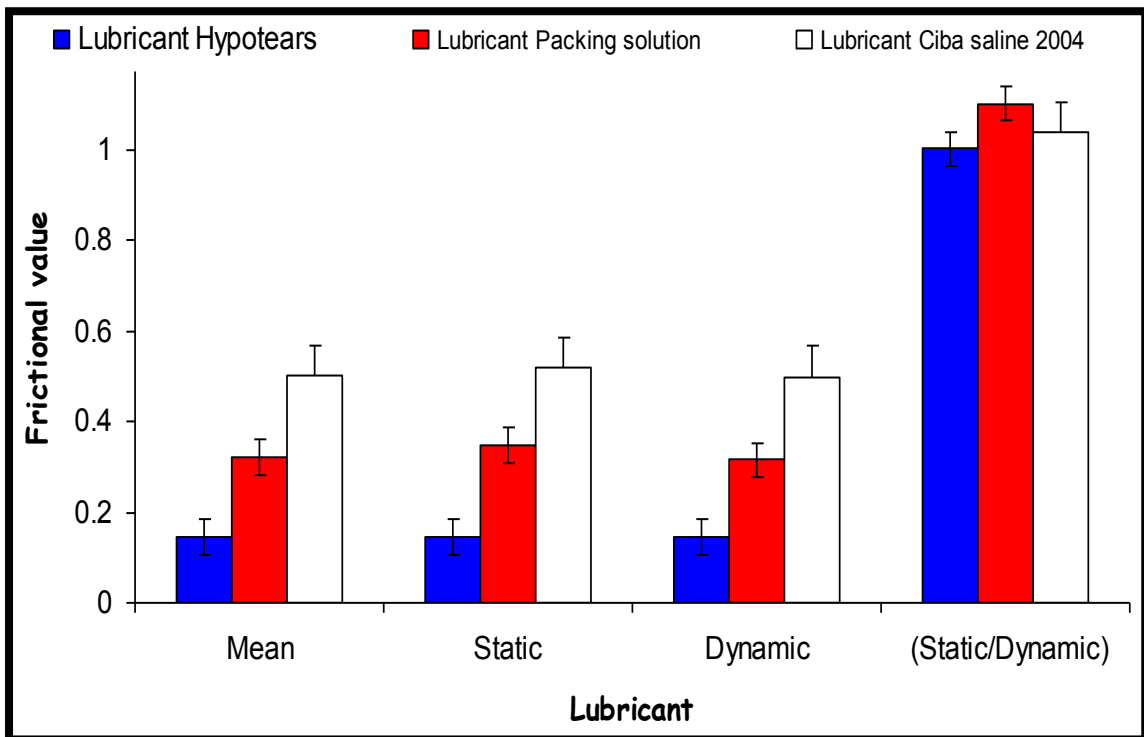


Figure 7.21. A summary of the frictional values of 7-day Safe-Gel™ with in combination with Ciba Saline, 7-Day Safe-Gel packing solution and HypoTears®.

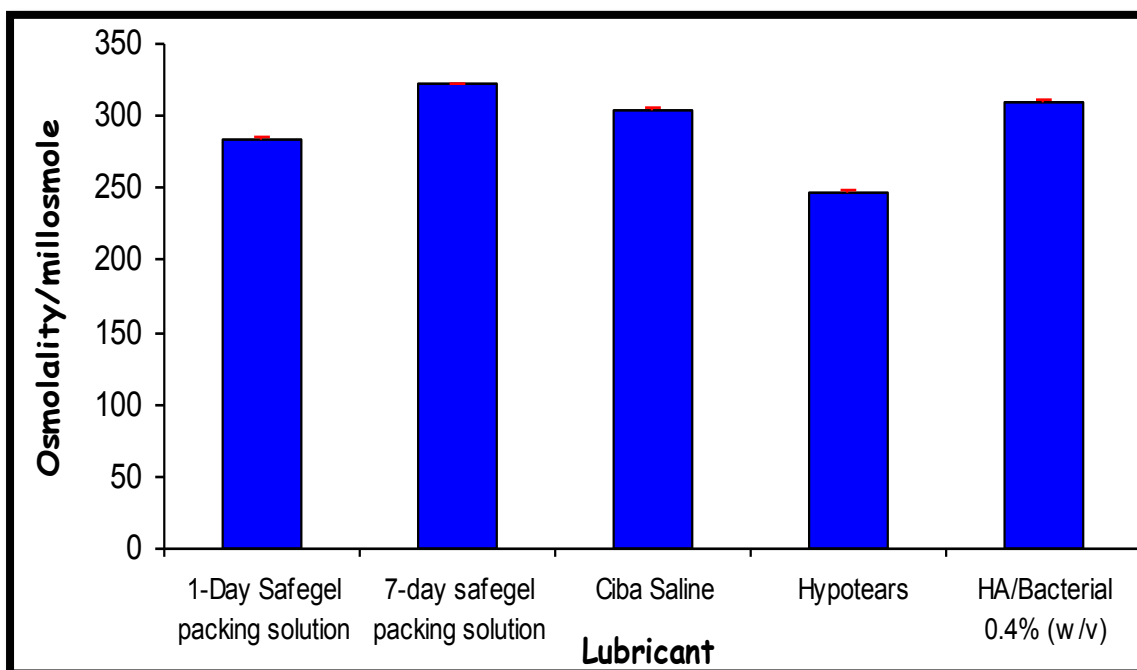


Figure 7.22. The osmolality of lubricants utilised.

### 7.3.2 Surface behaviour of lubricants via Langmuir studies

A 50 microlitre quantity of a 0.4% (w/v) bacterial fermentation sourced HA was injected onto a freshly cleaned water subphase surface at room temperature (23.2°C). Figure 7.23, demonstrates that HA does not show surface activity (the ability to adsorb onto a hydrophilic water subphase surface), at the air-water interface. It exhibits a collapse pressure of only 7mN/m. This would theoretically be equivalent to a dynamic surface tension of 65.8mN/m, under maximum pressure, in a set minimum area (72.8-7). See Chapter 5 for further details regarding surface measurements. This means that HA will not adsorb at an air-aqueous interface but instead will dissolve into the aqueous subphase bulk. Although, HA possesses a huge molecular weight, it shows no amphipathic nature, as it is an extremely hydrophilic macromolecule thus acquiring a strong tendency for dissolving well into the aqueous subphase bulk. In addition, this means that HA has no boundary lubricating, at an air-aqueous interface. This is strong evidence that HA is in fact, a fluid film lubricant, as described in chapter 1 by the Stribeck curve (Figure 1.5).

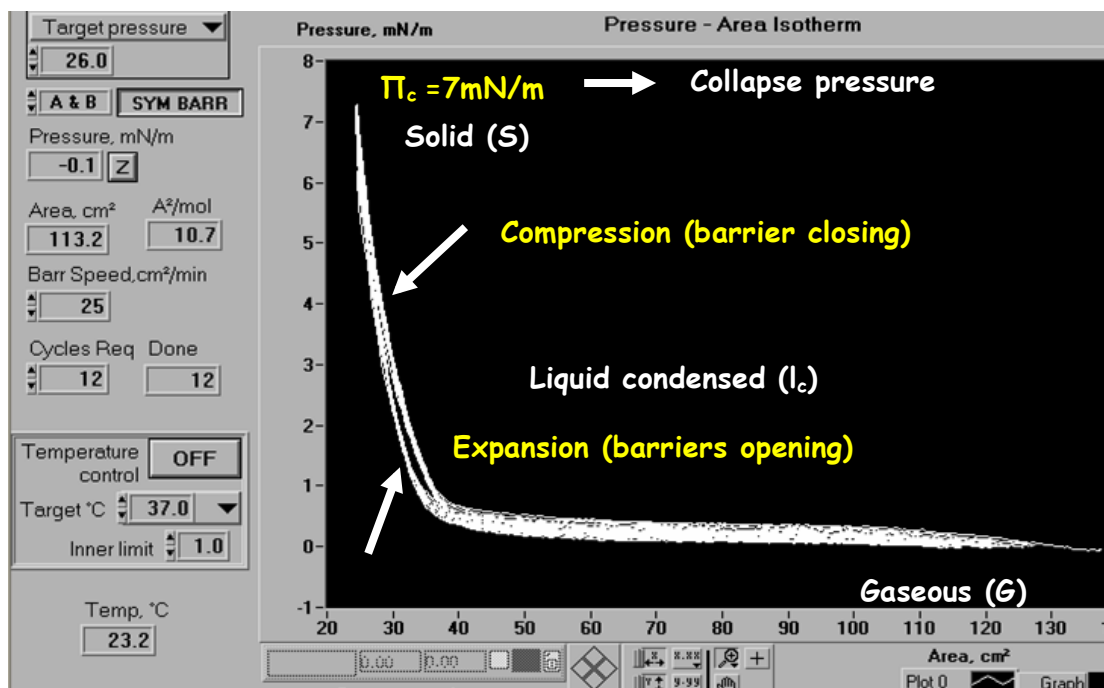


Figure 7.23. A Langmuir isotherm for 50 microlitres of 0.4% (w/v) HA bacterial fermentation with a collapse pressure of 7mN/m, at 23.2°C, exhibiting very little surface activity at the air-water interface, when spread at a speed of 25cm<sup>2</sup>/min.

However, surprisingly, the Langmuir isotherm of the 7-day Safe-Gel<sup>TM</sup> packing solution showed some mild surface activity (see Figure 7.24). The main features that are evident are; a slightly higher collapse pressure and a condensed Langmuir isotherm shape. What must be considered when interpreting this result is that the source of HA employed in Safe-Gel<sup>TM</sup> is HyaCare<sup>®</sup>.

This study has already highlighted the observation that the HA source affects its performance; this is further evidence to support this statement. Furthermore, there may be additional components (such as additives and buffering agents) included into the packing solution as well. This may also be responsible for the variation in surface behaviour observed.

Figure 7.24, presents the Langmuir isotherm of 50 microlitres of 7-day Safe-Gel<sup>TM</sup> packing solution that was injected onto a prior clean water subphase surface at room temperature (25°C). The shape of the isotherm was characteristic of a condensed Langmuir isotherm, showing a collapse pressure of 13mN/m, a dynamic surface tension of 59.8mN/m. The dictating factor in the difference, once again, may be due to the source of HA employed in Safe-Gel<sup>TM</sup> packing solution HyaCare<sup>®</sup>. Additionally, this may explain why a lower coefficient of friction is achieved. The most interesting finding in this work was the Langmuir isotherm of benzalkonium

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chloride (BAK) preserved HypoTears<sup>®</sup>. For an artificial tear, it showed a surprisingly high collapse pressure of 22mN/m under compression (see Figure 7.25). No other artificial tear exhibits such behaviour. Thus, the HypoTears<sup>®</sup> formulation is worthy of further careful consideration. Table 7.9 below summarises the preserved HypoTears<sup>®</sup> formulation.

Table 7.9. Composition of BAK preserved HypoTears®

<b>Active ingredient</b>	<b>Role</b>
1% PVA	lubricant
1% PEG 400	lubricant
0.1mg/ml BAK	preservative
dextrose	

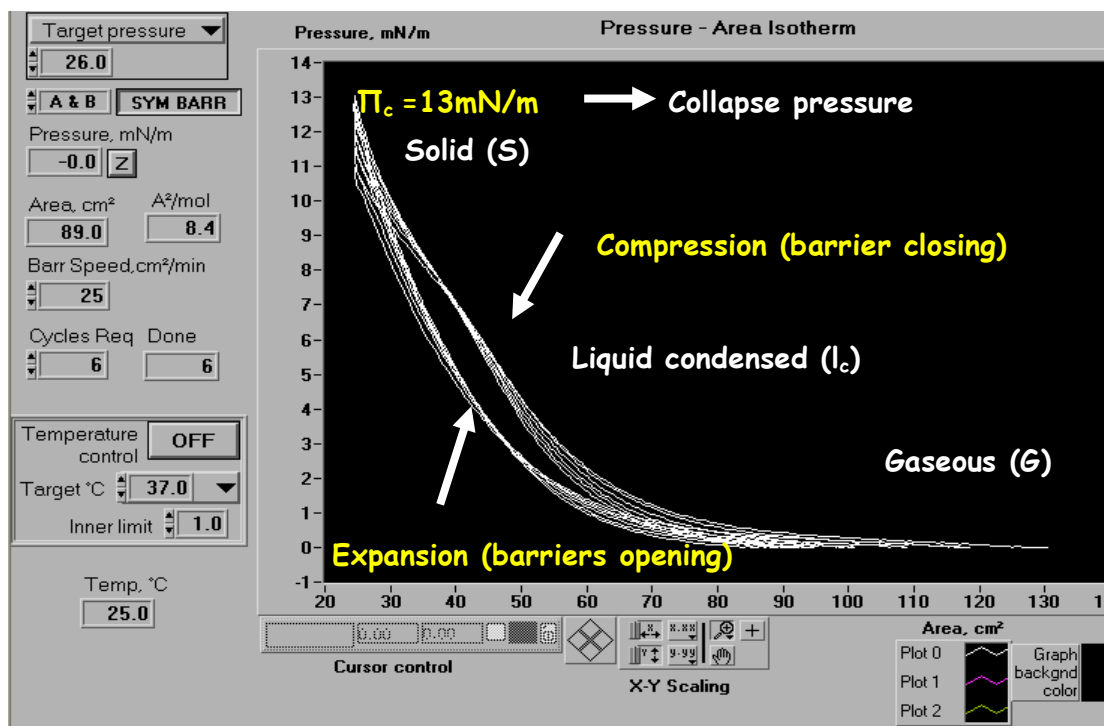


Figure 7.24. A Langmuir isotherm for 50 microlitres 7-Day Safe-Gel<sup>1M</sup>, demonstrating a collapse pressure of 13m N/m, a dynamic surface tension of 59 mN/m, under maximum compression, in the set minimum area, shows a condensed Langmuir isotherm at 25cm<sup>2</sup>/min.

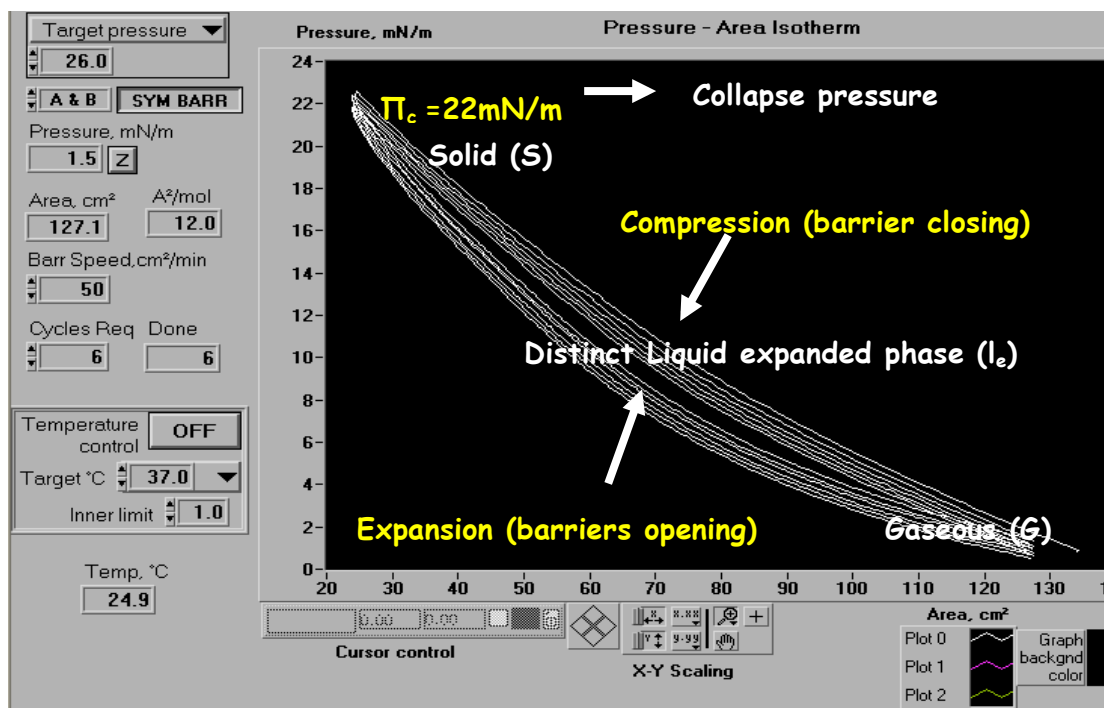


Figure 7.25. A Langmuir isotherm for 50 microlitres of HypoTears® demonstrating a collapse pressure of 22mN/m at 24.9°C, exhibiting an expanded Langmuir isotherm, with some hysteresis at 50cm<sup>2</sup>/min.

A 50 microlitre quantity of HypoTears® (BAK preserved) was injected onto a prior cleaned water subphase surface at room temperature (23.2°C), in drop-wise manner. A collapse pressure of 22mN/m, which would theoretically be equivalent to a dynamic surface tension of 50mN/m. This is remarkable surface activity for an artificial tear. The shape of the isotherm was typical of an expanded Langmuir isotherm, indicating that the HypoTears® formulation is surface active and thus has the ability to adsorb at the air-water interface. This may help us to understand why low coefficients of friction are always achieved whilst using HypoTears® as the lubricant. A distinct Langmuir expanded isotherm was visible (see Figure 7.25) suggesting that the injected solution was resisting some degree of compression, thus having the ability to remain at the air-water interface. This is due to the HypoTears® formulation; high molecular weight hydrophilic polymers such as PEG-400 and PVA (which show mild amphipathic nature), allows greater surface coverage due to the presence of BAK.



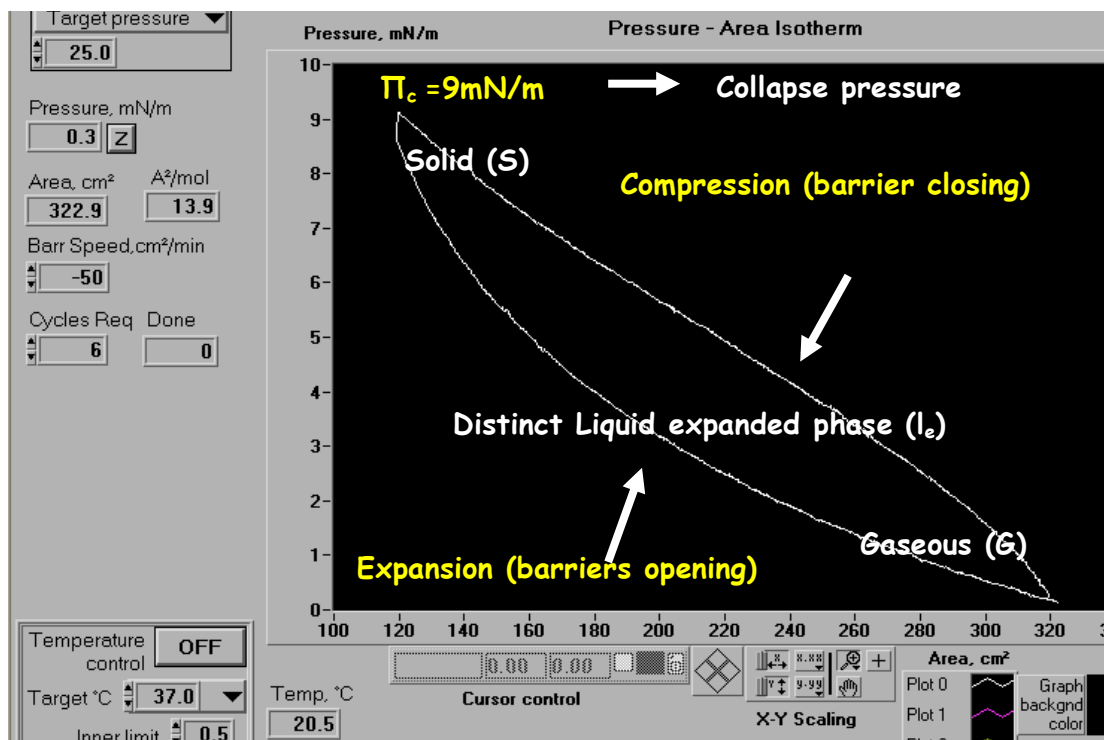


Figure 7.26. A Langmuir isotherm for 50 microlitres of HypoTears® unpreserved (without BAK) exhibiting a reduced collapse pressure of 9mN/m.

Figure 7.26 above, presents the Langmuir isotherm of non - preserved HypoTears®, even though the above isotherm was collected on a larger Langmuir trough (324cm<sup>2</sup>), Figure 7.26 depicts that the exclusion of BAK from the HypoTears® formulation results in a reduced collapse pressure (9mN/m), a reduction of the collapse pressure (at water subphase surface) by a factor of 2, but the distinct liquid expanded phase shape remained. Hysteresis was also still prominent. Additionally, estimating a higher surface tension of 63mN/m - under maximum area, in a set minimum area. This is strong evidence that the intriguing surface behaviour was due to the combination (a synergistic effect) of PVA, PEG and BAK, in commercially available preserved HypoTears®.

### 7.3.3 Hyal Safe-Gel™; A HA based multi-purpose solution [151-157]

It is interesting to investigate how HA behaves in different commercial products. HA has recently been introduced into ophthalmic multi-purpose solutions. Multi-purpose solutions, essentially consists of multi components - an antimicrobial, Polyhexamethylene Biguanide (PHMB) in this case, a buffering system (disodium edentate) and, in some cases, a surfactant (none in this case) and a lubricant for contact lens conditioning (HA in this case). Hyal Safe-Gel™ is a new multi-purpose

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solution with sodium hyaluronate. It is a simple formulation of PHMB, (0.001%); disodium edentate: 0.1%; HA 0.01% made up to 100% with excipients and purified water. It is surprising to note, that no additional surfactant was incorporated into the formulation.

### **7.3.4 Frictional behaviour of Hyal Safe-Gel™**

Figure 7.27, below illustrates that 1-day Safe-Gel™, in combination with Hyal Safe-Gel™ shows a lower coefficient of friction than with commercially prepared saline (Ciba saline) and Safe-Gel™ 1-day packing solution. Figure 7.28, reveals that an even lower coefficient of friction is attained with 7-day Safe-Gel™ and Hyal Safe-Gel™. This may also be due to the differences in water content of Safe-Gel™ material, and the concentration of HA incorporated into the 7-Day Safe-Gel™ matrix and packing solution.

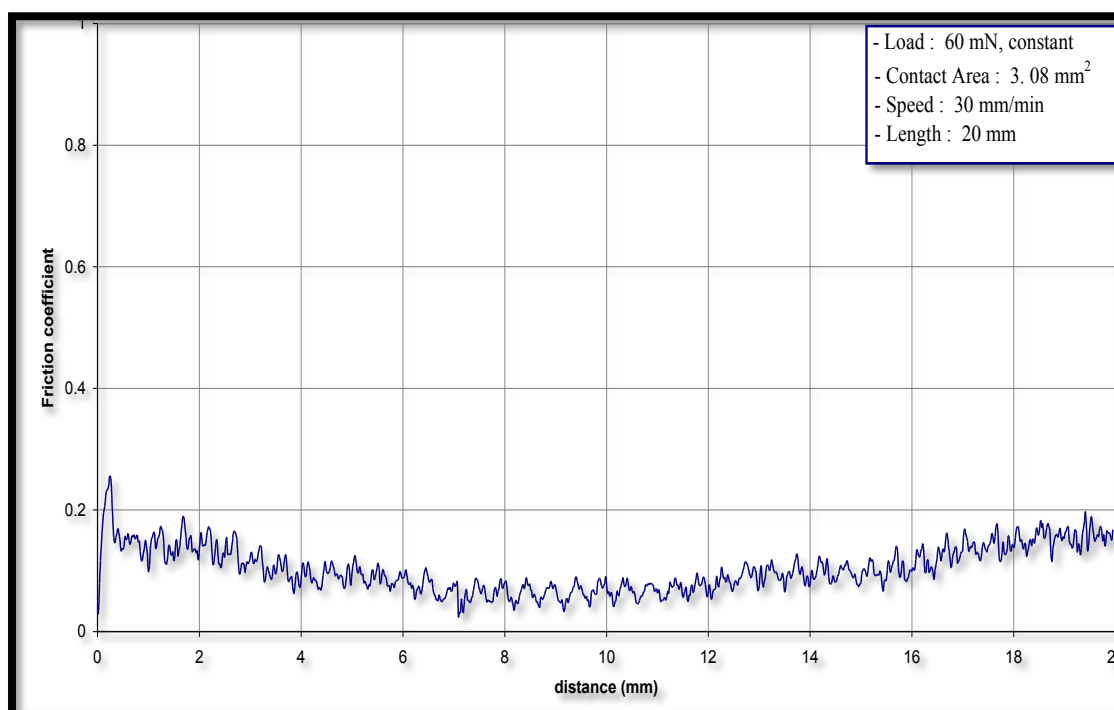


Figure 7.27. The coefficient of friction of 1-day Safe-Gel™ with distance travelled with Hyal Safe-Gel™ as the lubricant.

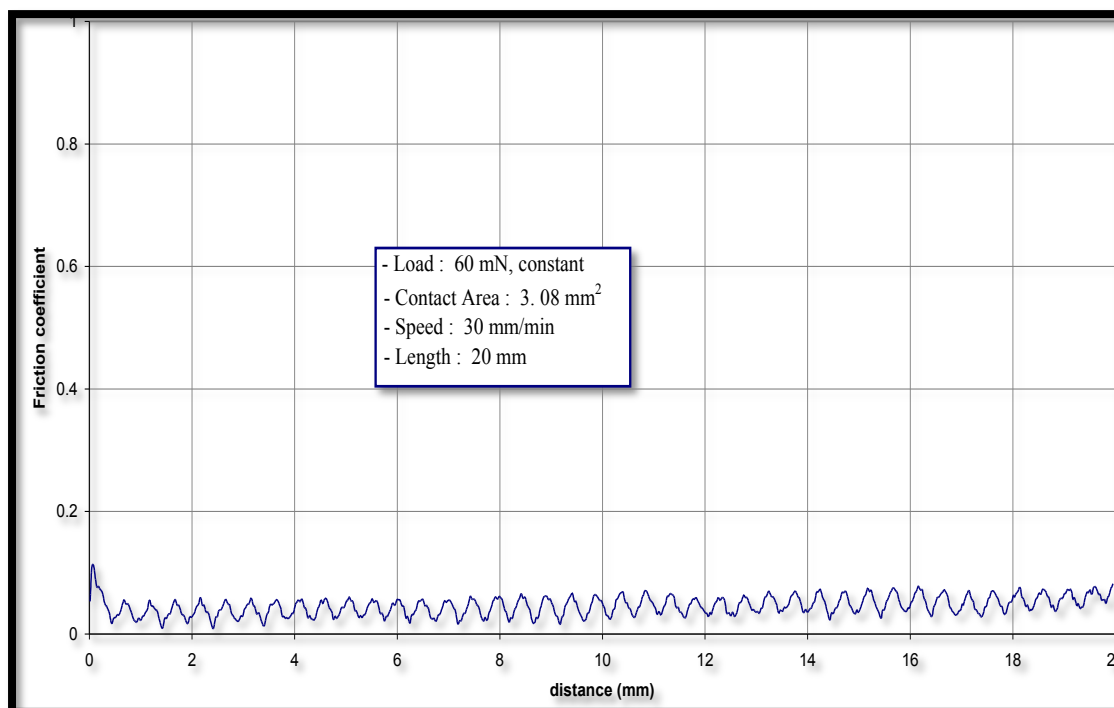


Figure 7.28. The coefficient of friction of 7-day Safe-Gel<sup>TM</sup> against distance travelled with Hyal Safe-Gel<sup>TM</sup> as the lubricant.

What can be inferred from all these measurements is that, ultimately the quality of lubricant (which possess the right chemistry) is the most dominating factor in achieving lower coefficients of friction and therefore comfortable contact lenses, in combination with HA-based ocular lubricants. Lower coefficients are observed with lubricants that are surface active, as this allows them to adsorb onto the contact lens surface, providing boundary lubricating capability too.

#### 7.4 Discussion

The role and mode of action of HA in ocular and articular joint lubrication has been discussed in this thesis. The elastic properties of the HA gel are reflected in its ability to act as a “shock absorber” for the eye. Its role in joint lubrication, as a major component of synovial fluid, is well recognised, where its viscoelastic properties enable it to lubricate the load bearing surfaces in a unique way. The long entangled molecules produce a high viscosity (lubrication) effect, which resists compression and also allows the joints to withstand tension and endure abuses, such as physical trauma and abrasion that other tissues in the body cannot withstand. Unfortunately, as the body ages HA production is impaired resulting in problems for both the joints and the eye. In consequence, HA (frequently in the form of its sodium salt) is

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increasingly introduced into these body sites to remedy this situation.

<sup>1</sup>H-NMR analysis revealed that the composition of HA copolymers vary with source. This will affect the performance of HA from different sources, i.e., the degree of hydrogen bonding with water. A 0.4% (w/v) of HA solution mimics the reported non-Newtonian, viscoelastic rheological behaviour of tears. The rooster comb source showed the most viscoelastic behaviour and the human umbilical cord source, the lowest coefficient of friction. This may be due to the varying molecular weights, purity and degrees of branching of the various HA copolymers. HA based viscosupplement injections are highly concentrated HA in buffer solution, some are crosslinked. The biological source of HA varies in the products, more contemporary products are based from bacterial fermentation sources, as opposed to rooster comb, which was previously favoured (despite it being of higher molecular weight), which is not as safe or as cheap. The more expensive products are of higher concentration as expected, due to the expense associated with HA purchase. Orthovisc, a highly concentrated HA solution [1%(w/v)] viscosupplementation treatment, exhibits a very low coefficient of friction (~0.09). The rheological behaviour of Orthovisc, however does not mimic the reported rheological behaviour of native synovial fluid. It appears to be very viscoelastic and shows Newtonian behaviour. As the shear rate is increased the viscosity of the solution does not decrease. HA visosupplementation shows no shear thinning behaviour. This may be a result of crosslinking a highly concentrated HA solution, which possess a great deal of chain entanglements, behaving as a single entity.

<sup>1</sup>H-NMR analysis of the highly viscous Orthovisc did not disclose any structural differences. So the difference in frictional and rheological is most likely due to concentration, molecular weight effects and more importantly due to the purity of HA in commercially available Orthovisc. This can further support the differences in rheological and frictional properties found for Orthovisc. A 0.4% (w/v) solution of umbilical cord HA exhibited the lowest coefficient of friction with etafilcon A. Contemporary literature states that although HA is a major component of synovial fluid, it is not solely responsible for the lubricating capability of synovial fluid. HA solutions possess no load bearing boundary lubrication potential unless incorporated with SAPLs. As HA solutions are viscoelastic, they provide hydrodynamic lubrication. The lubrication mechanism by which synovial fluid operates is in the mixed lubrication regime. In this lubrication mechanism the hydrodynamic and

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boundary lubrication occur simultaneously. The mechanisms are in competition and support each other in order to achieve the remarkable low coefficient of friction exhibited at a healthy synovial joint. Nature's load bearing boundary lubrication at articular joints is obtained from SAPLs.

The most profound reason why HA is employed into artificial tears is that is biocompatible and naturally present in the ocular site. Additionally, it is available more purely and at a lower cost today. HA artificial tears are dilute HA solutions, largely bacterial fermentation sourced. The attractive choice of HA into artificial tears is due its ability to generate viscous solutions at low concentrations. Of all the HA-based artificial tears tested, Blink<sup>®</sup> Contacts 0.15% (w/v) HA, (rooster comb sourced) exhibited the lowest osmolality, the most viscoelastic and the lowest coefficient of friction with etafilcon A contact lenses. Overall, HA-based artificial tears exhibited a higher frictional behaviour with etafilcon A ( $\geq 0.34$ ) than with nelfilcon A ( $\leq 0.15$ ). The difference between the two contact lenses is that the former is a charged substrate and the latter is a neutral hydrophilic substrate. These research findings suggest that HA is more likely to exhibit more effective lubricating effect with a hydrophilic neutral substrate.

The frictional behaviour of a contemporary HA based artificial tear Avizor moisture drops was lower with silicone hydrogel contact lens materials. Lower values were obtained with galyfilcon A (0.15) than with lotrafilcon B (0.25) - hydrophobic, silicone materials. The frictional behaviour of the HA-based contact lens (Safe-Gel<sup>™</sup>) was dependent on the lubricant utilised. HypoTears<sup>®</sup> appeared to be most compatible with both HA-based lenses and Ciba Saline the least. HypoTears<sup>®</sup> showed the most surface activity and exhibited the lowest osmolality of all the biolubricants studied. Ciba saline was the least surface active. The data obtained indicates that the source of HA, purity, concentration in solution and the molecular weight effects its performance. HA is a polyelectrolyte, combining it with salts and other actives in artificial tears will adopt its conformation and hence its properties. If HA is to be employed in contact lens solutions and lens materials, the concentration of HA, purity and molecular weight should be carefully selected, and it should be combined (most importantly) with a compatible surface active component.

A 0.4% (w/v) solution of bacterial fermentation HA (commercially utilised source) possesses very little surface activity at the air-water interface. Features of the

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Langmuir isotherm exhibited virtually no hysteresis and a collapse pressure of only ~ 7mN/m (a dynamic surface tension of 65mN/m). The mean osmolality of 0.4% (w/v) solution of bacterial fermentation HA was found to be 310 milliosmole. The 7-Day Safe-Gel<sup>TM</sup> contact lens system showed a lower coefficient of friction than the 1-Day Safe-Gel<sup>TM</sup> system, its packing solution may have had a higher concentration of HA (osmolality = 312) and was more surface active (collapse pressure of 13mN/m - a dynamic surface tension of 59.8mN/m) at the air-water interface. What can be inferred from all these measurements is that ultimately the quality of lubricant is the most dominating factor in achieving lower coefficients of friction. Lower coefficients are observed with lubricants that are surface active as this allows adsorption onto the contact lens surface, providing boundary lubrication as well as hydrodynamic, i.e., operating in the mixed lubrication regime. The HA based multipurpose solution Safe-Gel Hyal<sup>TM</sup> exhibited a low mean coefficient of friction with all the HA-based contact lenses. HA solutions provide hydrodynamic lubrication and not boundary lubrication, as they are viscoelastic and not surface active. Both lubrication mechanisms need to be in operation, in order to achieve lower coefficients of friction, and therefore attain comfortable HA-based contact lenses and effective HA-based ophthalmic solutions. Although, HA solutions lack boundary lubricating capability at the air-water interface (imparting a dynamic surface tension of 65mN/m), the inclusion of HA into ocular lubricants is beneficial due to its unique properties. The lens material that HA is integrated into must be carefully selected to maximise its benefits.

This study highlights that the cost of using HA in orthopaedic and specific ophthalmic lubrication applications, can be justified if HA is combined with surface active agents, such as SAPLs (nature's boundary lubricating agents). The challenge of combining HA with polar phospholipids has been successfully achieved in this thesis.

**CHAPTER 8:**  
**SUMMARY, DISCUSSION AND**  
**SUGGESTIONS FOR FURTHER WORK**

## **8.1 Summary**

The ultimate aim of the work carried out in this thesis was to develop novel biosurfactants based on HA, synthetic surfactant protein analogues and polar phospholipids, as an enhanced treatment to manage lubricant deficient diseases. The challenge was to examine the potential of combining HA with polar phospholipid (boundary lubricating agents) into clear solutions, without the usage of solvents. Contemporary literature recognises that HA solutions possess no load bearing boundary lubrication potential, unless incorporated with surface active phospholipids (SAPLs). The attempt therefore has been to investigate the potential of using and designing novel PSMA-polar phospholipid complexes, in order to improve the efficacy of HA containing biolubricants.

PSMA-based copolymers are known to undergo conformational transitions in response to environmental stimuli. This smart behaviour makes it possible to mimic the behaviour of native apoproteins. Native apoproteins (surfactant protein analogues) are well recognised as nature's lipid solubilising agents. In this work a biomimetic approach has been employed into seeking PSMA-based structures that generate lipid solubilising agents, which possess a critical pH that approaches physiological pH.

Two studies were thus devoted in, synthesising and characterising novel lipid solubilising agents based from PSMA-based copolymers, as synthetic surfactant protein analogues. The work undertaken in Chapters 4 and 5 was successful in furthering our understanding of the structure-property relationships for the contemporary PSMA-based copolymers sought. The work findings reveals that by varying the molecular weight, the maleic anhydride-to-styrene monomer ratio and more importantly, in the presence of additional (hydrophobic) mono-partial ester moieties, the pH-responsive behaviour of hydrolysed PSMA-based copolymers can be modulated, for specific application requirements. Chapter 4 identified unique PSMA-based structures and presented strong evidence for structure and microstructure, by NMR studies. The most interesting finding determined was that PSMA-based copolymers are not 100% perfectly alternating in contrast to what has been reported previously in the literature. Edited <sup>13</sup>C-NMR analysis was most informative in demonstrating the presence of regioisomerism in styrene-maleic anhydride systems. Additionally, this study provided some interesting insights into the polymerisation methodologies employed that exist for styrene and maleic



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anhydride polymerisation. The NMR spectra (for PSMA-based copolymers) were useful in revealing purity, the approximate styrene-maleic anhydride ratio and identifying additional mono-partial ester moieties, as reported by PSMA manufacturers.

In Chapter 5, novel hydrophilic and hydrophobic synthetic surfactant proteins have successfully been prepared from hydrolysing various PSMA-based copolymers. A whole range of hydrolysed PSMA-based solutions, from copolymers of maleic anhydride and styrene with different molecular weight, styrene-to-maleic anhydride monomer feed ratios (1:1 to 3:1) and with mono-partial methyl, propyl, isobutyl ester functionalities have been prepared. The method of hydrolysis was dependent on the copolymer type; however, all PSMA-based copolymers studied were successfully hydrolysed at 50°C with 1M sodium hydroxide solutions, within 48 hours.

Static surface tension analysis undertaken via the du Noüy ring detachment method demonstrated that hydrolysed PSMA-based solutions of varying molecular weight and HLB values, exhibit low static surface tensions of  $\leq 42\text{mN/m}$ . This provides evidence that they adsorb at the liquid-air interface and are in fact surface active, as expected. Langmuir studies were informative in demonstrating how the hydrophilic and hydrophobic polyanionic surfactants developed exhibit unique surface behaviour, at the air-aqueous interface. The trends observed, correlate well with the estimated theoretical HLB values. The aim of designing and synthesising novel synthetic surfactant protein analogues has been successfully achieved. Both hydrophilic and hydrophobic synthetic surfactant protein analogues have been synthesised. The PSMA MW 350,000 - mono-partial methyl ester 10-15% (w/v) purchased from S-A, in particular is worthy of discussion, due to the following desirable features:

- Ease of hydration and hydrolysis for polyanionic surfactant synthesis
- Desirable estimated HLB value - 17.5
- Mono-partial methyl ester moieties, effecting hydrogen bonding at critical pH
- Superior surface properties; low static and dynamic surface tension (unique Langmuir isotherm features) - dual functionality
- Lower coefficient of friction - viscous solution
- Lipid solubilising agent at a higher critical pH

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- Much higher molecular weight - allowing greater surface coverage (possessing a larger radius of gyration as MW = 350,000)

These features listed are desirable properties for an effective boundary lubricant. PSMA-based structures with these characteristics possess a dual function; they solubilise DLPC/DPPC at a higher critical pH, and additionally fulfil the criteria for a 'surfactant' protein analogue.

What can be concluded from the studies conducted in Chapter 5, is that a balance of these features are necessary in order to optimise the performance of a PSMA-based copolymer as effective novel lipid solubilising agents. The pH-responsive behaviour of PSMA-based copolymers is governed by achieving an appropriate balance of hydrophobic association and electrostatic repulsion.

The work carried out in Chapters 4 and 5 suggests that the pH- responsive behaviour is dictated by ;

1. Styrene and maleic anhydride polymerisation conditions - the monomer feed ratio will determine structure and microstructure. This has consequences on monomer sequence distribution, composition and molecular weight, as shown from the NMR data obtained in Chapter 4. This will largely effect the concentration of hydrophobic styrene, influencing the hydrophobic effect. In the case where a higher concentration of hydrophobic styrene is present, a greater interaction with the hydrophobic tails of polar phospholipids will be expected during PSMA-DLPC/DPPC association and PSMA-phospholipid complex formation.
2. Modification of maleic anhydride moieties, via esterification reaction with certain alcohols, will result in the presence of mono-partial ester with varying levels of esterification. This will influence the nature and strength of hydrogen bonding and reduce the strength of electrostatic repulsion by reducing the amount charge on the resulting polyanionic surfactant.
3. The method and degree of hydrolysis will determine the concentration of maleic acid moieties, which will in turn influence the electrostatic repulsion and nature and extent of the hydrogen bonding of the lipid solubilising agent prepared.

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By managing these factors, the pH-response of PSMA-based copolymers can be modulated and lipid solubilising agents, which possess a critical pH approaching physiological pH, can be designed and thus synthesised.

In conclusion, the ideal critical pH of the hydrolysed PSMA-based solutions for the application this thesis is concerned with, would be at physiological pH. The precise definition for the target structure, HLB value,  $pK_a$  therefore warrants further investigation and development.

Furthermore, synthetic surfactant analogues can be designed by varying the hydrophilic and hydrophobic balance, ultimately by forming more effective surface active agents. Although, hydrolysed PSMA-based copolymers are generally weak polyacids, the  $pK_a$  can be adjusted by altering the styrene and maleic acid ratio. The pH range over which a reversible phase transition occurs, can be generally modulated by changing the critical pH, by incorporating a hydrophobic moiety into the polymer backbone. The challenge is therefore to achieve a balance of good hydration (enough hydrophilic moieties) and additionally to adjust the  $pK_a$  such that the critical pH of the target lipid solubilising agent approaches that towards physiological pH. So, essentially weaker polyacids will exhibit a higher critical pH, a lower HLB value and in turn more surface active polyanionic surfactant behaviour. The critical pH for 1:1 molar PSMA-based copolymers is at very acidic conditions (pH~3). These copolymers have some limitations, having an estimated HLB value of around 26-28. The preparation of clear solutions from these materials, and hence macromolecular assemblies require a lowering of the pH to between 3-5. This is often undesirable.

In light of all the data obtained in this PhD work regarding PSMA-based copolymers, in order of importance, the following factors affect the pH-responsive behaviour;

- Presence of mono-partial esters and thus degree of esterification
- Hydrolysis method and efficiency for polyanionic surfactant synthesis
- Styrene-to-maleic anhydride monomer feed ratio
- Molecular weight of PSMA-based copolymer

The presence of hydrophobic mono-partial ester moieties has the greatest consequences on the surface properties. The hydrophobic and hydrophilic balance

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is changed. Not only is the charge density reduced of the PSMA-based copolymer, but the nature and extent of the hydrogen bonding is affected. See Chapter 5 for a detailed explanation of these phenomena.

The Astosomes technology developed at Aston allows organised solubilisation of polar phospholipids, to form clear solutions with the use of lipid solubilising agents. This approach is a unique selling point and superior in comparison to other attempts of Lipoidal supplementation which involve solvents and use animal based products. These products are also associated with allergy risks and toxicity issues.

The materials developed in this thesis (see Chapter 6) show amazing surface properties, exhibiting both low static and dynamic surface tensions. Novel lipid solubilising agents have been prepared making unique formulations that are more stable and are effective at the target physiological pH. Furthermore, these assemblies are temperature resistant and can be freeze dried to prolong shelf life and therefore have promising potential for commercialisation in biomedical applications. The synthesis temperatures vary due to the differences in the phase temperature of phospholipids, and on the pH clearing range (the critical pH). This was dependent on the lipid solubilising agent employed. These formulations combine well with HA solutions, satisfying the main objective of this work.

Langmuir studies (once again) have proved most useful, illustrating that the choice of lipid solubilising agent employed for biosurfactant synthesis affects the surface behaviour. The surface properties of these materials approach that of extracted native Lipoidal films, which are promising and strongly suggest that these materials are very suited for ocular lubrication applications.

BAM was used for the first time to visualise the novel PSMA-DLPC complexes, at the air water interface. BAM analysis illustrates that the PSMA-DLPC complexes adsorb and form aggregates at the air-aqueous interface (at room temperature). As the complexes are visible this provides strong evidence that they form microstructures as well. This indicates that the size of the PSMA-DLPC complexes, have a population (range) from microstructures to nanostructures.

Rheological analysis discloses that the combined HA and PSMA-DLPC complexes exhibit a rheological behaviour that mimics the non-Newtonian behaviour of native

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biosurfactants. The biosurfactants developed in this work have certain limitations; they lack good frictional and rheological properties, they are poor hydrodynamic lubricants. They are small molecules. These limitations can be resolved by combining the biosurfactants with a large macromolecule, such as HA (a perfect candidate).

HA is unique and a very important molecule in nature, and thus justifies why it is well researched in the field of biomaterials science. HA is biocompatible, hydrophilic, cellular healing, a large macromolecule that shows viscoelastic behaviour (shear thinning behaviour, at the right concentration). It is well known for being a good hydrodynamic lubricant. Above all, it is economically feasible today to employ HA now, as it is cheaper, purer and also safer. Furthermore, it is readily available in an ultra-pure form known as - HyaCare<sup>®</sup>. However, HAs limitations, as a lubricant in orthopaedic and ophthalmic applications are that it has no boundary lubricating capability, and shows poor surface behaviour at the air-aqueous interface. These shortcomings can be resolved by combining HA-based solutions with PSMA-DLPC complexes - surface active agents (boundary lubricants).

### **8.1.1 Concluding remarks**

In conclusion (in light of the entire thesis findings) the combined PSMA-DLPC/DPPC-HA complexes, exhibit a balance of *in-vitro* frictional, rheological, surface properties that are composition dependent and show competitive advantage, as novel biosurfactants (synthetic biological lubricants). The synergistic effect of the two solutions is greater than the additive effects. Further investigation is therefore necessary to enhance the biosurfactants developed and explore the potential for commercialisation. Additionally, the formulations developed need to be tailor made and thus optimised for specific applications requirements. However, it should be noted that these materials exhibit *in-vitro* surface properties that approach the *in-vitro* surface properties of native Lipoidal films. This strongly suggests that these biomaterials are more likely to be successful and useful in ophthalmic biolubrication applications, in particular for managing patients with dysfunctional tear films due to lipid deficient dry eye states.

## **8.2 Discussion**

Although, Mother Nature provides many inspirations for designing and developing new materials, creating synthetic systems capable of responding to stimuli in a controllable and predictable fashion represents significant challenges. Particular challenges lie in mimicking biological systems where structural and compositional gradients at various length scales are necessary for orchestrated and orderly responsive behaviour. In this work, the strategic approach undertaken for designing and synthesising novel lipid solubilising agents was achieved using functional biomimesis. PSMA-based copolymers must be carefully selected for specific application requirements. These materials should be designed by careful polymerisation techniques to meet target specifications. Due to the presence of anhydride functionalities in the polymer system styrene-maleic anhydride copolymer materials can be modified. Much chemical modification work of the styrene-maleic anhydride copolymer has been done in order to change its degree of hydrophilicity and other properties. By creating charged maleic acid groups, PSMA-based copolymers are solubilised into water, which is achieved through hydrogen bonding effects and charge. As the maleic acid functionality dictates the pH-responsive behaviour, it is vital that the efficiency in which the maleic anhydride is converted into maleic acid groups is thus optimised. In this thesis, time was thus devoted in ensuring that the conditions for PSMA-based copolymers hydrolysis is enhanced and carefully defined.

The approximate value of the copolymer composition - monomer molar ratio, can be calculated from  $^1\text{H-NMR}$  integral peaks data. However, a more accurate quantitative analysis can be obtained by measuring the  $T_1$  values for the polymer under study, (so correct time delay can be set) when collecting the FID (free induction decay).

For the lower molecular weight PSMA-based solutions studied, longer hydrolysis time (at  $50^\circ\text{C}$  for 48 hours) leads to the preparation of a more surface active PSMA-based solutions. This is due to the greater concentration of maleic acid group that can ionise into the water achieving a more effective hydrophobic-hydrophilic balance. PSMA MW 9,500 with higher styrene content (3:1 monomer feed ratio) is more surface active than the lower molecular PSMA MW 1,600 (1:1 monomer feed ratio). This demonstrates that the surface properties of PSMA-based copolymers are controlled by varying the molecular weight and styrene and maleic content (i.e., the

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HLB value). Higher concentrations of high molecular weight PSMA-based solutions demonstrate viscous solutions and Newtonian rheological behaviour. This can be a limiting factor when measuring the static surface tension of such solutions via the du Noüy ring detachment method. However, such solutions can provide effective hydrodynamic lubricants. The PSMA MW 350, 000 - mono-partial methyl ester (10-15% w/v) has a lower static and dynamic surface tension; in contrast to other PSMA-based copolymers, that exhibit higher dynamic surface tensions. The most useful finding from *in-vitro* surface characterisation is that hydrolysed PSMA MW 350, 000 - mono-partial methyl ester (10-15% w/v) demonstrates a superior balance of surface properties.

Of all the PSMA-based copolymers studied, NR6 was the easiest to hydrate and hydrolyse, despite it being a much higher molecular weight. Furthermore, it possesses an estimated HLB value of 17.5, which achieves a good balance of surfactant behaviour and solubilisation characteristics. This meets the desired specification requirements for a novel lipid solubilising agent for boundary biolubrication applications. Additionally, the higher molecular weight allows greater surface coverage, being another attractive feature. The key therefore is that a balance of properties is required for an effective lipid solubilising agent (synthetic surfactant protein analogue) in biolubrication applications.

PSMA-based copolymers are class of hydrophobically associating polymers that have the ability to form flattened disk like molecular assemblies with lipids. Copolymers of styrene and maleic anhydride acid with a 1:1 styrene-to-maleic anhydride ratio (hydrolysed styrene/maleic anhydride copolymers have a  $\text{PK}_a$  value of 3.75-4). The  $\text{pK}_a$  for the individual acid values being 1.97 and 6.24, respectively. Preparation of clear solutions and hence macromolecular assemblies require a lowering of the pH to around 3-5. Such pH levels are not suitable for compositions, which are to be applied to sensitive surface of the body. Although, the pH of these copolymer formulations may be raised after the formation of the polymer phospholipid complex, such adjustment tend to lead to instability, which may be observed as a loss of clarity over time as the macromolecular assemblies degrade. There is a need to produce a stable, non-irritating formulating aid that enables oil-soluble active agents incorporated into aqueous media at high concentration, while at the same time forming macromolecular complexes that are small enough not to disrupt the passage of light through the resultant solution, i.e., to remain substantially

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clear. The work in this thesis has made a great starting point to achieve this.

Due to the range of motion that occurs at the biological surface interface, Lipoidal material plays a crucial boundary biolubrication protecting role. It is nature's load bearing boundary lubricant and has the capability of adsorbing and retaining at the biological interface. It can essentially operate in the mixed lubrication regime. Lipids are insoluble in aqueous media, strong evidence in the literature addresses that the interaction of lipids with proteins in nature renders them aqueous soluble (through hydrophobic interactions). Lipid supplementation has already been commercially carried out to manage lipid deficient diseases such as dry eye and respiratory distress syndrome, but no attempt has been made to treat OA. The addition of a FDA approved surfactant (Pluronic NF127) enhances the lipid solubilisation (loading) with certain hydrolysed PSMA-DLPC combinations, and prolongs the shelf life of certain formulations. PSMA-DLPC complexes can be freeze dried to prolong their shelf life. The PSMA-DLPC based complexes exhibit an amazing low static surface tension (26mN/m) via the du Noüy method and a high surface activity at the air-water interface with Langmuir studies (collapse pressure of >45mN/m was observed). The static surface tension values concurred well with the dynamic surface tension values attained, via Langmuir studies.

The collapse pressure of the PSMA-DLPC complexes can be enhanced with the usage of a higher molecular weight, more hydrophobic; PSMA MW 350, 000 - 10-15% (w/v), mono-partial methyl ester and the blocky PSMA MW 7, 500 (St: MA monomer feed ratio of 3:1) lipid solubilising agents. These novel lipid solubilising agents prepared have a HLB value of 17.5 and 14 respectively. The use of higher molecular weight and more hydrophobic lipid solubilising agents enhances the adsorption of the PSMA-DLPC complexes, at the air water interface. This being evident in the features of Langmuir pressure-area isotherm generated. A greater hysteresis and a higher collapse pressure of 50mN/m are reached, denoting a low dynamic surface tension of 22mN/m under maximum compression, in a minimum area, as the PSMA-DLPC films are squeezed. These PSMA-DLPC complexes also portray a more distinct liquid expanded phase, which indicates they are more resistant to compression, suggesting a varied self assembly at the air-water interface, in comparison to the behaviour of PSMA-DLPC complexes prepared from using lower molecular weight hydrophilic PSMA-based lipid solubilising agents. The presence of mono-partial ester moieties changes the self assembly.



HA is a mucopolysaccharide occurring widely in nature. HA solutions provide hydrodynamic lubrication and not boundary lubrication, as they are viscoelastic and not surface active. Both lubrication mechanisms need to be in operation in order to achieve lower coefficients of friction and therefore effective HA-based solutions. In conclusion, the cost of using HA in biolubrication applications can be justified if its surface properties are improved or if it is combined with compatible surface-active components. The lubrication mechanism by which biological lubricants operates is in the mixed lubrication regime. What can be inferred from the all thesis findings is that ultimately the quality of lubricant is the most important factor in achieving lower coefficients of friction. Lower coefficients are observed with lubricants that are surface active, as this allows them to adsorb onto a surface, providing boundary lubrication too, when the hydrodynamic lubrication breaks down. The novel biosurfactants developed can operate in the mixed lubrication regime and satisfy thus the requirements for effective biomimetic synthetic lubricants.

### **8.3 Suggestions for further work**

#### **8.3.1 NMR studies**

Calculate  $T_1$  (longitudinal relaxation) values for all PSMA-based copolymers, so quantitative NMR analysis can be conducted. This will allow accurate polymer composition quantification. NMR spectra acquired using different deuterated solvents (and at higher temperature) may improve the problem of the poor NMR resolution spectra, associated with PSMA-based copolymers obtained in this thesis. Quantitative  $^{13}\text{C}$  spectra should be carried out on all hydrolysed PSMA-based solutions, firstly to provide structural evidence for hydrolysis of the maleic anhydride moieties. Furthermore, in order to also secondly actually quantify the extent of hydrolysis. This will allow quantitative analysis for the ease and extent of hydrolysis, for each PSMA-based copolymer studied. Quantitative carbon (Dept 135) can be conducted to quantify the degree of regioisomerism and the ratio of the 'triad' sequence for all PSMA-based copolymers sought. Advanced 2D NMR analysis, would additionally reveal further insight into the complex microstructure and quantify monomer sequence distribution, for PSMA-based copolymers. Phosphorous NMR of all the PSMA-DLPC/DPPC complex combinations (at various pH ranges) would provide useful information regarding the differences in structural self assembly (chemical environment) for all the various novel biosurfactants developed.

### **8.3.2 PSMA-Phospholipid complex synthesis**

RAFT polymerisation extends the microstructure range for the styrene-maleic anhydride copolymers that are available for hydrolysis and modification. Factors that affect the responsive behaviour of hydrolysed PSMA-based solution are microstructure; molecular weight, stereochemistry, monomer sequence distribution and polydispersity index (PDI). This is because the radius of gyration is PDI and molecular weight dependent. Natural polymers such as proteins and DNA have specific secondary structures, defined architecture and are monodisperse. RAFT polymerisation allows for direct synthesis of many polyampholytic block copolymers without the need for protecting group chemistry. Copolymerisation of styrene and maleic anhydride can be performed under living polymer conditions, something considered impossible until now. It will be also necessary to control the interfacial chain lengths, molecular weights, and chain stiffness. Thus, incorporating well defined responsive copolymers with a desired PDI into the interfacial regions, using the precision of RAFT polymerisations may be essential in the future. New RAFT polymerisation reactions will broaden the range of styrene-maleic anhydride copolymers that are available for hydrolysis. The PDI, molecular weight and microstructure of PSMA-based copolymers should be tailored and thus fine-tuned, for specific application requirements. Improvements on hydrolysis conditions will further optimise polyanionic surfactant synthesis, define and enhance hydrolysis methodology. Modified PSMA-based copolymers, (esterification with various alcohols) will generate unique PSMA-based structures, to advance our understanding of structure-property relationships. The possibility of freeze drying the HA along with other co-agents such GAGs and co-surfactants (i.e., Pluronic NF127) with PSMA-DLPC/DPPC needs to be explored and optimised for improved biosurfactant synthesis. Stabilisation strategies need to be conducted in order to prolong the shelf life and define the storage conditions. Phase diagrams need to be constructed for the contemporary hydrolysed PSMA-based copolymers, which possess a broader HLB value range. This will establish if the Lipoidal loading can be enhanced by carefully selecting the required lipid solubilising agent. Investigation of complexing the various hydrolysed PSMA-based solutions with other Lipoidal species and other GAGs (other than HA) would be interesting. The possibility and advantages of using more than one lipid solubilising agent (both a hydrophobic and a hydrophilic synthetic surfactant protein analogue) should be studied, as nature has both in operation, working together synergistically.

### **8.3.3 Surface chemistry**

The determination of  $pK_a$ , will accurately define the critical pH range of all the novel synthetic protein analogues (lipid solubilising agents) developed. It would also be useful to measure the zeta potential and the PDI of the various PSMA-based copolymers. Techniques such as transmission electron microscopy, atomic force microscopy and scanning electron microscopy would provide useful information regarding the structure, morphology, and size of the HA-PSMA-DLPC/DPPC complexes developed. Langmuir and BAM provide valuable information concerning the surface properties of biolubricants. In future, this technique has great potential for assessing the effectiveness of boundary lubricants, at the air-water interface. The reflectivity number for the film images obtained (via BAM) would be useful to quantitatively analyse the various films (biosurfactants) developed.

As biological interfaces are animated rather than static such as in the eye during blinking, the lungs during breathing and the joints during articular joint movement, it is therefore necessary to measure the surface properties of the biosurfactants developed under dynamic conditions, to mimic *in-vivo* conditions. This can be achieved by carrying out dynamic surface compression measurements with a pulsating bubble surfactometer (PBS). Advanced frictional studies, at high load and at low shear (for orthopaedic applications) need to be conducted and also investigating the effects of different substrates is necessary.

HyaCare<sup>®</sup> branded HA (by Novozymes) has been reported in the literature to exhibit superior properties compared to other commercial HA brands. HyaCare<sup>®</sup> should be tested in order to investigate the reliability of these claims, by carrying out analytical techniques (already described in this thesis) which have been utilised to assess other various biological sources of HA. HA is a polyelectrolyte combining it with salts and actives in artificial tears will adopt its conformation and hence performance. If it is utilised in contact lens materials, the lens material that HA is integrated into must be carefully chosen to maximise its benefits (for HA supplementation) towards contact lens development. HA would be most cost effective to incorporate into the back (posterior) surface of a contact lens, as opposed to integrating it into the matrix in order to provide cushioning and thereby solid hydrodynamic lubrication. Additionally, it will hydrate the posterior contact lens surface due to its hydrophilic nature. Finally, the stability and performance of these novel synthetic biomimetic lubricants must be

optimised. More importantly, the biosurfactants developed need to be tailored for specific application requirements and also tested under specific *in-vitro* conditions, to assess performance for target specifications.

#### **8.4 Future Directions**

Although, fair progress has been achieved for developing new lubricious materials which exhibit better *in-vitro* performance than the existing materials, below is a list of potential projects that can be undertaken to further enhance the materials developed in this work, and to additionally make better use of the work carried out in the thesis:

- Explore the incorporation of the polymer-phospholipid complexes into contact lens materials, towards the development of novel biomimetic contact lens materials.
- Employ the polymer-phospholipid complexes, as conditioning agents for contact lenses comfort and care solutions.
- Develop biomimetic artificial tears for management of dry eye symptoms.
- Enhance a biomimetic synthetic synovial fluid for viscosupplementation needed for OA pain management.
- Develop novel delivery systems for loading and delivering hydrophobic matter for drug delivery applications.

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